Mode of action of FMRFamide-like peptide on *Drosophila* body wall muscle contractions

by

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ABSTRACT

Neuropeptides are the largest group of signalling chemicals that can convey the information from the brain to the cells of all tissues. DPKQDFMRFamide, a member of one of the largest families of neuropeptides, FMRFamide-like peptides, has modulatory effects on nerve-evoked contractions of *Drosophila* body wall muscles (Hewes et al., 1998) which are at least in part mediated by the ability of the peptide to enhance neurotransmitter release from the presynaptic terminal (Hewes et al., 1998, Dunn & Mercier., 2005). However, DPKQDFMRFamide is also able to act directly on *Drosophila* body wall muscles by inducing contractions which require the influx of extracellular Ca^{2+} (Clark et al., 2008). The present study was aimed at identifying which proteins, including the membrane-bound receptor and second messenger molecules, are involved in mechanisms mediating this myotropic effect of the peptide. DPKQDFMRFamide-induced contractions were reduced by 70% and 90%, respectively, in larvae in which FMRFamide G-protein coupled receptor gene (CG2114) was silenced either ubiquitously or specifically in muscle tissue, when compared to the response of the control larvae in which the expression of the same gene was not manipulated. Using an enzyme immunoassay (EIA) method, it was determined that at concentrations of 1 µM- 0.01 µM, the peptide failed to increase cAMP and cGMP levels in *Drosophila* body wall muscles. In addition, the physiological effect of DPKQDFMRFamide at a threshold dose was not potentiated by 3-Isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, nor was the response to 1 µM peptide blocked or reduced by inhibitors of cAMP-dependent or cGMP-dependent protein kinases. The response to DPKQDFMRFamide was not affected

in the mutants of the phosholipase C-β (PLCβ) gene (norpA larvae) or IP_3 receptor mutants, which suggested that the PLC-IP_3 pathway is not involved in mediating the peptide's effects. Ala1 transgenic flies lacking activity of calcium/calmodulin-dependent protein kinase (CamKII) showed an increase in muscle tonus following the application of 1 μM DPKQDFMRFamide similar to the control larvae. Heat shock treatment potentiated the response to DPKQDFMRFamide in both ala1 and control flies by approximately 150 and 100 % from a non heat-shocked larvae, respectively. Furthermore, a CaMKII inhibitor, KN-93, did not affect the ability of peptide to increase muscle tonus. Thus, although DPKQDFMRFamide acts through a G-protein coupled FMRFamide receptor, it does not appear to act via cAMP, cGMP, IP3, PLC or CaMKII. The mechanism through which the FMRFamide receptor acts remains to be determined.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AC	adenyl cyclase
AMP	adenosine monophosphate
ATP	adenosine-5'-triphosphate
cAMP	3',5'-cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase, type 2
CCAP	crustacean cardioactive peptide
cGMP	3',5'-cyclic guanosine monophosphate
DAG	diacyl glycerol
dFMRFamide	*Drosophila* FMRFamide
DFR	*Drosophila* FMRFamide receptor
DMSR	*Drosophila* myosuppressin receptor
Drm-MS	*Drosophila melanogaster* myosuppressins
Drm-SK	*Drosophila melanogaster* sulfakinins
EJP	excitatory junctional potential
FR	FMRFamide receptor
G-proteins	guanine nucleotide-binding proteins
GC	guanylate cyclase
GPCR	G-protein coupled receptor
GDP	guanosine diphosphate
GMP	guanosine monophosphate
GTP	guanosine-5'-triphosphate
IBMX	3-isobutyl- 1 -methyl-xanthine; a non-selective phosphodiesterase inhibitor
IP_3	inositol 1,4,5-triphosphate
MS	myosuppressins
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLC	phospholypase C
PP_i	pyrophosphate
Rp-cAMPS	adenosine 3′,5′-cyclic monophosphorothioate, Rp-Isomer, triethylammonium salt; PKA inhibitor
Rp-8-pCPT-cGMPS	guanosine 3′,5′-cyclic monophosphorothioate, 8-(4-Chlorophenylthio)-, Rp-Isomer, triethylammonium salt ; PKG inhibitor
RYR	ryanodine receptors
SK	sulfakinins
UAS	upstream activation sequence
VGCC	voltage-gated Ca^{2+} channels

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Brief outline of the matter

The survival of an organism depends on its ability to monitor its external environment and to adapt its behaviour in response to changes in the environment. This is evident even in the life of simple unicellular eukaryotes such as *Dictyostelium discoideum*, a slime mould. When exposed to starvation, cells of *D. discoideum* secrete a chemoattractant, cAMP, that acts on neighbouring cells, presumably directly through cAMP receptors (Devrotes, 1983; Henderson, 1975). When neighbouring cells detect extracellular cAMP they move toward the source. At the same time, intracellular cAMP levels in cells that received the signal increase, which is followed by the secretion of more cAMP into the extracellular environment and, consequently, the chemoattracting signal propagates to other cells. As a result, several thousand cells are able to aggregate forming a multicellular fruiting body (Gerish, 1982; Deverotes, 1983). Although chemical signalling in multicellular organisms has evolved strikingly from that in unicellular organisms, it is still similar in the way that the response to external cues is a result of the ability of individual cells, specialized to carry out a specific physiological function, to coordinate their activities. Hence, unicellular and multicellular organisms developed systems by which cells can communicate with each other to produce integrated physiological and behavioural responses.

A major class of chemical signals, neuropeptides, are chemical signals that convey the information from one nerve cell to another nerve cell, or to a cell of a distant non-neuronal tissue. This allows for the regulation of various developmental and

physiological processes and processes involved in generating animal behaviour. Hence, the disruption in either synthesis or function of neuropeptides can lead to a disruption of individual processes and overall functioning of an organism. Based on their common structure, neuropeptides are grouped into families. Often the structure and function of members of individual families have been conserved throughout the animal kingdom. Several families of neuropeptides are classified as myotropic peptides based on their ability to affect muscle contractions. One of the largest and most extensively investigated families of myotropic neuropeptides is the FMRFamide (Phe-Met-Arg-Phe-NH_2)–like family of peptides (FLPs) that share structural similarity to a cardioexcitatory peptide FMRFamide, first isolated from molluscan bivalve, *Macrocallista nimbosa* clam by Price and Greenberg (1977). Since then, numerous peptides with the carboxyl terminus RFamide (Arg-Phe-NH_2) were identified in vertebrates and invertebrates and were found to influence a variety of physiological processes such as synaptic output and muscle contractility (Espinoza et al., 2000; Nassel, 2002; Nichols, 2003, Mercier et al., 2003). These peptides were referred to as FMRFamide-related peptides (FaRPs; Price & Greenberg, 1989) for over a decade. Since some RFamides (notably the myosuppressins and the FMRFamides) are not evolutionarily related and act on different G-protein coupled receptors (GPCRs), it has been recommended that the term "FaRP" be abandoned to avoid confusion (Scholler et al., 2005). Therefore, in the current study, FLP will refer to all the peptides whose carboxyl terminus ends in FMRFamide (Phe-Met-Arg-Phe-NH_2), while the term RFamides will encompass all the other peptides including myosuppressins and sulfakinins.

Recently, it has been shown that the *Drosophila* FMRFamide-like peptide, DPKQDFMRFamide, enhances nerve-evoked muscle contractions (Hewes et al., 1998) and synaptic transmission in the neuromuscular junction (Hewes et al., 1998, Dunn & Mercier, 2006). It also modulates muscle contractility by directly inducing muscle contractions and increasing muscle tonus (Clark et al., 2008), but the exact mechanisms through which the peptide is acting are not known. The present study was designed with the goal to elucidate the transduction mechanisms underlying the effects of this specific *Drosophila* FMRFamide-like peptide which may give us more insight in how the peptides of this family act in general.

1.2 Why study molecular aspects of chemical signalling?

Much work in all areas of biology has been devoted to studying the molecular mechanisms of chemical signalling, but it is often asked why one should study signalling networks and, more specifically, what is the significance of studying signalling networks in insects, such as *Drosophila*. First, chemical signalling mechanisms are present in the simplest unicellular eukaryotes (e.g flagellata), and the presence of enzymes sharing homology with human enzymes, such as adenylate cyclase and insulin degrading enzymes in bacteria, suggests that the roots of chemical signalling go beyond eukaryotes and may have evolved in prokaryotic organisms (Pertseva, 1991). Some molecular components of signalling pathways (i.e. adenylate cyclase and G proteins) have kept their main function throughout evolution, suggesting that the organisation and the properties of molecular particulates of chemical signalling are similar across all animal phyla. Hence, the knowledge gained from research on chemical signalling and its

molecular components in insects such as *Drosophila melanogaster* can be relevant for other animals. Secondly, disruptions or changes in chemical signalling cascades have been related to a number of diseases, such as atherosclerosis, asthma, diabetes, mood disorders and cancer. Thus, it is not surprising that chemicals involved in signal transduction pathways emerge as top targets for drug treatments. The identification of potential drug targets relies greatly on what is known about the physiology of the cell. Thirdly, broadening our knowledge about chemical signalling and its molecular components will contribute to understanding the basic physiology of organisms, and aid in understanding how signals flow within cells, and what are the chemical messengers that propagate information from one cell to another to generate specific activities and behaviours.

In general, two main approaches are taken when studying the signal transduction pathways: pharmacological and genetic. An array of pharmacological agents has been identified to activate or disrupt specific molecular components of signalling pathways. In the genetic approach, *Drosophila* has been proven to be a great model system. Since the entire *Drosophila* genome is sequenced, a large number of genes that encode for molecular components involved in signal transduction have been identified. With the development of molecular techniques, many fly lines with mutations in such genes became available. Newer techniques can be used to overexpress or knockdown relevant genes in a temporal and tissue-specific pattern.

1.3 Neuropeptides: a major class of chemical signals

Chemical signals are often classified into groups based on their range of action and their structural similarities. Chemical signals are thought to act in three ways: (a) on the cell that releases the chemical signal (autocrine signalling), (b) on the cell of a neighbouring tissue (paracrine signalling) and (c) on the cell of a distant tissue (endocrine signalling). Neural systems utilize all three types of signalling to affect the physiological function of virtually all cells through the actions of two main classes of chemical signals, small molecule neurotransmitters and neuropeptides (Connors, 2003; Kandel et al., 1995). Small molecule neurotransmitters include amino acids and their derivatives (glutamate, aspartate, GABA and glycine) and monoamines (acetylcholine, serotonin, dopamine, epinephrine and histamine). Neurotransmitters are released from the nerve terminal and diffuse a short distance across the synaptic cleft (approx. 50 nm) to bind to receptors on a postsynaptic cell or, in some cases, to a receptor on the presynaptic terminal itself. Hence, they have a role in paracrine and autocrine signalling. Neuroendocrine signalling involves release of chemicals that act on distant targets (Roman, 2003; Kandel et al., 1995).

Neuropeptides are a diverse group of oligopeptides that co-exist and are co-released with small molecule neurotransmitters at the synapse (O' Shea & Schaffer, 1985; Hokfelt, 1991; Hokfelt et al., 2000). They can act as neurotransmitters themselves, or they can modulate the actions of other neurotransmitters. But, many neuropeptides are also present in neurosecretory cells, from which they can be secreted into the circulatory system and carried to distant targets. Thus, neuropeptides can also function as hormones (Hokfelt et al., 2000; Taghert & Veenstra, 2003). The presence of the

neuropeptides in the central and peripheral nervous system has been conserved from invertebrates to mammals (Hokfelt et al., 2000; DeLoof & Schoofs, 1990). The presence of some vertebrate neuropetides (e.g β–Endorphin, insulin, gonadotropin) in unicellular organisms suggests that they have an enormous significance among chemical messengers. The great structural and functional diversity of an enormously large number of neuropeptides led to the grouping of peptides with common amino-acid sequences into families of neuropeptides. To date, crustacean and insect neuropeptides have been classified into 22 distinct families (Mercier et al., 2007), although the discovery of novel peptides is not yet excluded. Among others, their physiological functions include regulation of development (preecdysis triggering hormone, prothoracicotropic hormone, ecdysis triggering hormone, corazonin, insulin-like peptide and eclosion hormone), metabolism (adipokinetic hormone), water absorption (corticotrophin releasing factor-like peptide, calcitonin-like diuretic hormone, leucokinins, arginine-vasopressin like peptide and neuroparsins) and muscle tension (allostatins and allotropins, cardioactive peptides, FMRFamides, leucokinins, pyrokin related peptides, tachykinin-related peptides, SIFamide peptide, proctolin and orkokinins). The mechanisms of action of neuropeptides, with the emphasis on myotropic peptides and more specifically FMRFamide-like peptides (FLPs), are discussed in more detail below.

1.4 Signal transduction

In general, intercellular chemical communication can be divided into several steps (Krauss, 2001; Roman, 2003). In some cases, a presynaptic cell secrets a

transmitter that binds directly to an ionotropic receptor, which directly alters the flow of ions through a channel. In the case of metabotropic receptors, the following steps occur:

1. synthesis, release and transport of a chemical signal to a target cell,
2. recognition of the signal by a receptor on the target cell,
3. transduction of the external signal into an intracellular signal, which is accomplished by the activation of enzymes that produce specific second messengers,
4. second-messenger-dependant activation of appropriate effectors, such as enzymes, ion channels and transcription factors,
5. further transformation of reactions catalyzed by the modified effectors into a physiological response.

The specificity of the cell`s response to a given signal is maintained by the ability of the activated receptor to stimulate a specific second messenger pathway. These secondary messenger molecules are also important for amplifying a signal, since one signal molecule can induce production of a number of second messenger molecules that can in turn activate an even greater number of secondary effectors (Krauss et al., 2001; Roman, 2003). However, the ability of a cell to respond to a specific chemical signal in the first place depends on whether that cell contains receptors that will recognize the signal.

Two main types of receptors that have been shown to mediate the effects of neuropeptides are ionotropic and G-protein coupled receptors (GPCRs). Their general

structure and function is described below with more detailed emphasis on the receptors mediating responses to peptides belonging to RF-amide family.

1.4.1 Ionotropic receptors

Classical or small molecule neurotransmitters produce very rapid responses, which is mainly attributed to their action via ligand-gated ion channels. Binding of the neurotransmitter to the receptor directly opens an ion channel and allows movement of ions across the cell membrane. An example of this type of receptor is the nicotinic acetylcholine receptor, a 290 kDa transmembrane spanning protein complex that functions as an ion channel. Upon the binding of acetylcholine, the receptor undergoes a conformational change which rotates M2 helices and opens the channel that passes Na^+ and K^+ ions across the cell membrane (Kandel et al., 1995).

In general, neuropeptides act via G-protein coupled receptors that are associated with second messenger pathways. However, the first peptide ionotropic receptor was identified in *Helix aspersa* as an amiloride-sensitive sodium channel that was shown to mediate responses to FMRFamide (Phe-Met-Arg-Phe-NH_2) (Green et al. 1994; Cottrell, 1997, Lingueglia et al., 1995). Green et al (1994) reported that FMRFamide depolarized C2 neurons in *Helix aspersa* by activating two inward currents. One was a large, rapidly developing and rapidly desensitizing current, blocked by amiloride (an inhibitor of Na^+/ Ca^{2+} exchanger), suggesting that FMRFamide acts by modifying sodium currents. The currents were rapidly activated in inside-out patch recordings by 10 and 50 μM FMRFamide, even in the presence of 100 μM GDP [β-S], which inhibits binding of GTP to G-protein and hence, inhibits GPCR activation. These data suggested that the response

to FMRFamide is not mediated by G proteins and second messengers but results from direct binding of the peptide to sodium channels (Green et al., 1994). The gene encoding for this FMRFamide receptor (*FaNaCh*) was then cloned from a *Helix aspersa* cDNA library using a 310 bp fragment for amiloride-sensitive epithelial Na^+ channel proteins, *ENaChs* (Lingueglia et al., 1995). The cloned *FaNaCh* DNA predicted a protein with two hydrophobic membrane spanning domains and a long extracellular loop with a cysteine rich region. These sections show similarity to the cloned subunits of a Na^+ channel (*ENaChs*) on the apical surface of epithelial cells in the kidney, distal colon and lung of mammals, and to an amiloride-sensitive channel of *C. elegans* (Cottrell, G.A.,1997). No structural similarity to acetylcholine, glycine, GABA, glutamate and serotonin receptors was observed (Lingueglia et al., 1995). The exact physiological functions of the *FaNaCh* and its role in synaptic transmission are still to be investigated.

1.4.2 G-protein coupled receptors (GPCRs)

Most neuropeptides exert physiological effects primarily through GPCRs. GPCRs are among the primary targets for drug treatments. Genes encoding GPCRs constitute a large group that can take up to 1-2% of the animal genome (Hauser et al., 2006). In humans more than 1000 GPCR genes have been identified, which comprises about 3% of the human genome (Vauquelin & von Mentzer, 2007). GPCRs are transmembrane (TM) spanning proteins that consist of seven membrane spanning α helices (TM domains) which are interconnected with three intracellular and three extracellular loops. The amino (N) terminus is located on the extracellular side, whereas the carboxylic (C) terminus is located on the cytoplasmic side. Both the N-terminus and TM domains

contain amino acid sequences that are recognizable by specific ligands and, therefore, play a critical role in ligand binding (Vauquelin & von Mentzer, 2007; Meeusen et al., 2003). However, because of the large size of peptide messengers, the binding site for these molecules is likely to be near the N terminus (Flower et al., 1999). The C terminus contains several phosphorylation sites that can play a role in receptor desensitization.

GPCRs have been divided into several classes based on their structural similarities: (a) family A, rhodopsin-like receptors, (b) family B, secretin/glucagons/VIP family, (c) family C, metabotropic glutamate receptors and (d) family D, the fungal pheromone family. Most neuropeptides act via members of the family A, the rhodopsin-like receptors. The members of this family share a conserved arginine sequence in the Asp/Glu-Arg-Tyr motif of the third transmembrane helix (Mercier et al., 2007; Vauquelin & von Mentzer, 2007; Meeusen et al., 2003). Only about 10 % of neuropeptides act via receptors of family B, which are implicated in regulating processes such as growth, nutrition absorption, and cell proliferation (Vauquelin & von Mentzer, 2007). The structural similarity of the members of this family is in the conserved cysteine residues and multiple glycosilation sites on the N terminus end.

The common characteristic of all G-protein coupled receptors in the signalling pathway is binding to GTP-binding proteins (G-proteins), membrane-associated proteins that act as messengers between receptors and their target intracellular effectors. In its inactive state, a G protein is a heterotrimer consisting of α, β and γ subunits. The α subunit is usually hydrophilic and is bound to guanosine diphosphate (GDP), while the β and γ subunits form a $\beta\gamma$ complex that has a role in anchoring the G-proteins to the

plasma membrane. Upon binding of a ligand to its receptor, the receptor associates with the G-protein, which facilitates the exchange of GDP to GTP on the α subunit. This leads to dissociation of the GTP-bound α subunit from the receptor and from the $\beta\gamma$ complex. Both, the free GTP-bound α subunit and the released $\beta\gamma$ complex can associate with and stimulate different enzymes (e.g. adenylate cyclase (AC), guanylate cyclase (GC) and phospholipases A_2 and C) and, hence, modulate the production of second messenger signals, or they can act directly on ion channels. Based on the sequence of α subunits and their function, G proteins are grouped into four classes: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$. It is generally accepted that the class of G_s proteins stimulate adenylate cyclase, which then catalyzes the conversion of ATP into cAMP and pyrophosphate (PP_i), whereas the G_i class of proteins inhibits AC activity. Activation of the $G_{i/o}$ family of G proteins also stimulates phospholipase A_2, which further releases arachidonic acid (AA) from a membrane phospholipid. On the other hand, $G_{q/11}$ proteins activate a completely different enzyme, phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into two secondary messengers, diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3).

In addition to α subunits, $\beta\gamma$ dimers can also influence several effectors. The $\beta\gamma$ subunits released from activated G_i affect phospholipase C activity (Gudermann et al., 1996) although less potently than the α subunit (Camps et al., 1992). $\beta\gamma$ subunits also directly affect ion channels. For example activation of muscarinic, opioid or somatostatin receptors and subsequent activation of $G_i/_o$ G-proteins leads to the activation of K^+ channels and to the inhibition of Ca^{2+} channels through direct binding by the $\beta\gamma$ complex (Dascal, N.,2001; Jiang & Bajpayee, 2009).

1.4.2.1 RFamide GPCRs

RFamide and FLP GPCRs have been identified in several animal phyla. A GPCR (GRL106) activated by the RFamide cardioexcitatory peptide LyCEP (TPHWRPQGRF-NH2) was cloned from the mollusc *Lymnaea stagnalis* (Tensen et al.,1988). Eleven GPCRs that respond to RFamide ligands were identified from the nematode *C. elegans* (McVeigh et al. 2006). Radioligand binding assays revealed that the RFamide receptor from the optic lobe of squid (*Loligo pealei*) has a high binding affinity for FMRFamide and is coupled to G_s protein (Chin et al.1994). Similarly, Wang et al. (1995) found evidence that YDVDHVFLRFamide inhibitory effects on locust oviducts are mediated via GPCR since the increase in concentration of GTPγS, a nonhydrolyzable GTP analog, reduced the binding of the receptors to YDVDHVFLRFamide. The failure of cholera toxin and pertusis toxin to affect the specific binding of the receptors to YDVDHVFLRFamide suggested that this receptor is not coupled to either G_s or G_i (Wang et al., 1995).

GPCRs for *D. melanogaster* myosuppressins (Drm-MS), sulfakinins (Drm-SK) and FMRFamides (FLPs) have also been identified and cloned. cDNA sequences revealed that the myosuppressin GPCR (DMSR-1) gene CG8985 is 3,286 nucleotides (nt) long and consists of five exons and four introns, while the myosuppressin GPCR (DMSR-2) gene CG13803 is 2,618 nt long and has four exons and three introns (Egerod et al., 2003). The cDNA of the *Drosophila* FMRFamide receptor (DFR) gene CG2114 is 3,061 nt long, consisting of 2 exons and 1 intron (Cazzamali & Grimmelikhuijzen, 2002). Northern blot analysis revealed differences between the mRNA expression of cloned receptors, DMSR-1 (CG8985), DMSR-2 (CG13803) and DFR (CG2114). While Egerod et al (2003) reported

very weak mRNA expression of the two DMS receptors in embryos, larvae, and pupae, Cazzamali and Grimmelikhuijzen (2002) found that the DFR transcript, present in all of the developing stages of *Drosophila*, had the most pronounced expression in larvae and adults. In adult flies, DMSR-1 and DMSR-2 transcripts were strongly expressed in the head, but only DMSR-2 was present in the adult body. More detailed information about the DFR expression pattern in organs of wandering stage larvae was obtained with RT-PCR analysis, which revealed that the receptor is present in several Drm larval organs, including trachea, brain, gut, fat body, and Malpighian tubule (Meeusen et al., 2002).

The effects of a number of different peptides on transfected Chinese hamster ovary (CHO) cells expressing cloned DMSR-1, DMSR-2 or DFR were tested using an aequorin bioluminescence assay that detects changes in intracellular Ca^{2+}. Two independent studies, Meeusen et al. (2002) and Cazzamali and Grimmelikhuijzen (2002) tested the effects of FLPs and other RFamides on the DFR expressed in CHO cells. Both studies revealed that the intrinsic *Drosophila* FMRFamide peptides activated the DFR receptor, although there are discrepancies in the order of their potency. In both studies, it was found that Drm-FMRFamide-5 (PDNFMRF-NH_2) was the most potent in activating DFR (Table 1). Following the PDFMRFamide, intrinsic FMRFamides activated DFR receptor in the following order (from highest to the lowest potency,): DPKQDFMRFamide > TPAEDFMRFamide> SPKQDFMRFamide, SDNFMRFamide according to Cazzamali & Grimmelikhuijzen (2002), or SDNFMRFamide > DPKQDFMRFamide > SPKQDFMRFamide > TPAEDFMRFamide as per Meeusen et al (2002). (Table 1). It is interesting to note that SAPQDFVRSamide failed to activate the receptor (Cazzamali &

Grimmelikhuijzen, 2002) suggesting that the RFamide sequence at the N terminal is necessary for the peptide-receptor binding to occur.

The FMRFamide receptor can also bind peptides that are structurally similar to FMRFamides but with EC_{50} values higher than those peptides containing FMRFamide N terminal sequence (e.g. 2×10^{-7} M for Drm-MS; Meeusen et al., 2002;

Table 1. FLP effects on dFMRFamide GPCR expressed in Chinese hamster ovary cells

Peptide	**Sequence**	**EC_{50} (nM)** (a)	**~EC_{50} (nM)** (b)
Drm-FMRFamide-1	DPKQDFMRF-NH_2	2.0	3
Drm-FMRFamide-2	TPAEDFMRF-NH_2	2.8	9
Drm-FMRFamide-3	SDNFMRF-NH_2	1.9	500
Drm-FMRFamide-4	SPKQDFMRF-NH_2	2.5	9
Drm-FMRFamide-5	PDNFMRF-NH_2	1.8	0.9
Drm-SK-1	FDDYGHMRF-NH_2	38	-
Drm-SK-2	GGDDQFDDYGHMRF-NH_2	105	-
Drm-MS	TDVDHVFLRF-NH_2	91	200
FMRFamide	FMRF- NH_2	28	6
RFamide	RF-NH_2	n.a (>10uM)	-

a) Meeusen et al., 2002
b) Cazzamali & Grimmelikhuijzen, 2002
- not tested

Cazzamali & Grimmelikhuijzen, 2002). Hence, the FMRFamide receptor appears to be a true FMRFamide receptor that can differentiate between different N terminal amino acid sequences and is less likely to be a genuine receptor for other -RFamide peptides,

although it is possible that peptides such as Drm-MS may bind to the receptor under unusual physiological conditions with high peptide levels.

CHO cell lines transfected with cDNA coding for either DMSR-1 (CG8985) or DMSR-2 (CG13803) responded to DMS with an EC_{50} of 4 x10^{-8} M, whereas other peptides, crustacean cardioactive peptide capa-1, -2, and -3,corazonin, adipokinetic hormone, tachykinin-3, short neuropeptide F-1, ecdysis triggering hormones-1 and -2, pigment dispersing hormone, pyrokinin-2, drostatins-A4 -B2, and –C, and FMRFamide, failed to induce a response in the concentrations up to 10^{-6} M or 10^{-5} M (Egerod et al., 2003). Similar results were reported by Johnson et al. (2003), where simultaneous transfection of HEK-293 cells with a β-arrestin molecule tagged with a green fluorescent protein (Barr-GFP) and the GPCR of interest was used as a method to visualize the activation of GPCRs. This method is based on the desensitization mechanisms shared by all GPCRs, which assumes that all GPCRs are inactivated by two types of proteins, GPCR kinases (GRKs) and β -arrestins. When the receptor is in its inactive state, β-arrestins are diffused in the cytoplasm, but upon the activation of GPCR the β -arrestins are recruited and translocated to the membrane. The cells expressing *CG13803* responded to 1 μM DMS by the translocation of Barr-2, but did not respond to 1 μM DPKQDFMRF-amide. Comparing the actions of DM and DPKQDFMRFamide, Johnson et al (2003) found Drm-MS to be more potent in inhibiting forskolin-induced cAMP production in *CG13803*- HEK-293 and *CG898*-HEK-293 cells (EC_{50}: 1.7 x 10^{-10} M and 1.8 x 10^{-9} M respectively) than DPKQDFMRFamide (EC_{50}: 4.5 x 10^{-9} M and 1.3 x 10^{-8} M respectively). The ability of peptides to inhibit cAMP production also suggests that DMSRs may be coupled to $G_i/_o$

proteins. Cells expressing the dFMRF-amide receptor encoded by *CG2114* showed Barr2-GFP translocation in response to the 1 μM DPKQDFMRF-amide and Drm-MS, which would be predicted based on the result reported by Cazzamali and Grimmelikhuijzen (2002) (Table 1). However, the dose dependent effects of these peptides on β -arrestin translocation were not established, and, therefore no inference can be made about the relative sensitivities of the CG2114 to two different peptides.

Finally, the *Drosophila* SK receptor (DSKR-1) has also been cloned and pharmacologically characterized. Sulphated [Leu7]-DSK-1 increased the Ca^{2+} signal in HEK-293 cells transfected with DSKR1 in a dose-dependent manner (EC_{50}= 5.3 nM) (Kubiak et al., 2002). The effect was not affected by pertussis toxin incubation, which suggests that this receptor does not activate $G_i/_o$ proteins.

To summarize, RFamides can exert their effects via both GPCRs and ionotropic receptors. It appears that three different groups of RFamides (*Drosophila* FMRFamides, myossuppresins and sulfaknins) act through different GPCRs, all of which were cloned and identified in *Drosophila* (Meeusen et al., 2002, Cazzamalli et al., 2002, Johnon et al, 2002, Kubiak, 2002, Egerod et al., 2003). To date, however, functional characterization of these GPCRs was confined to cell cultures, either Chinese hamster ovary cells or Human embryonic kidney cells. Studies using this approach reported that one of these GPCRs, FR (gene CG2114), binds with high affinity to FMRFamides, while it was less sensitive to other RFamide peptides (Meeusen et al., 2002; Cazzamali & Grimmelikhuijzen, 2002). It follows that if the physiological effect of DPKQDFMRFamide

on the larval body wall muscle contracture was mediated by a GPCR, it is likely to be mediated by the DFR.

1.5 Myotropic neuropeptides

Approximately half of the identified insect neuropeptides have stimulatory or inhibitory effect on muscle tissue and are referred to as myotropic neuropeptides (Mercier et al., 2007; Taghert & Veenstra, 2003). Although, a large proportion of research has been devoted to investigating the mechanisms of peptide-induced modulation of insect muscle contractility, the knowledge is still very limited. Some of the most investigated myotropic neuropeptides include crustacean cardioactive peptide, proctolin and FLPs, and in the next section I will briefly outline their physiological effects on crustacean and insect muscles and what is known about the mode of their action.

1.5.1 CCAP

Crustacean cardioactive peptide (CCAP; Pro–Phe–Cys–Asn–Ala–Phe–Thr–Gly–Cys-amide) was first isolated as a crustacean cardiostimulatory peptide of the crab, *Carcinus maenas* (Stangier et al., 1987). Subsequently, it was shown that CCAP stimulates contractions of the locust spermathecal (da Silva & Lange, 2006) oviduct (Donini et al., 2001) and hindgut (Donini et al., 2002;Stangier, 1988) tissues. CCAP increased the amplitude of nerve-evoked contractions, basal tonus, and the frequency of spontaneous contractions of the spermatheca (da Silva & Lange, 2006) and oviducts (Donini et al., 2001, 2002). In the absence of nerve stimulation, spermathecal and oviduct muscles responded to CCAP with a threshold concentration of approximately 1 nM; the maximum effect occurred at 100 nM for spermathecal muscles and 500 nM for

oviduct tissue (da Silva & Lange, 2006; Donini et al., 2001). CCAP -induced contractions in locust oviducts were abolished in calcium-free saline containing 0.1 mM EGTA (a calcium chelator) and in saline containing 20 mM cobalt chloride, (a calcium channel blocker) indicating that extracellular Ca^{2+} is necessary for the response to occur (Donini et al., 2002). When applied individually, L-type and T-type calcium channel blockers reduced the tonic component of CCAP-induced contractions by approximately 30-50%. This suggests that the peptide induces Ca^{2+} entry via voltage-gated Ca^{2+} channels (VGCC). However, the peptide increased the tonus of oviduct muscles even after the VGCC were activated via high potassium saline-induced membrane depolarization, which suggests that, in addition to VGCCs, the peptide also uses non-voltage gated Ca^{2+} channels (Non-VGCCs) (Donini et al., 2002). The peptide also stimulates Ca^{2+} release from internal stores, since 10 µM TMB-8, an intracellular calcium antagonist, reduced the tonic component of 10^{-8} M CCAP-induced oviduct contractions by 30-35 % and delayed the time to reach the contraction peak (Donini et al., 2002).

1.5.2 Proctolin

Proctolin is a pentapeptide (H-Arg-Tyr-Leu-Pro-Thr-Oh) isolated and identified as a myostimulatory peptide in the hindgut of the cockroach *Periplaneta americana* (Brown & Starrat, 1975). Proctolin is associated with controlling visceral and skeletal muscle contractility in insects (Orchard et al., 1989) and hindgut tissue of crustaceans (Mercier et al., 2007; Mercier & Lee, 2002).

A large number of studies focused on elucidating the mechanism underlying proctolin myotropic action. In the mandibular closer muscles of the locust, *Locusta*

*migrat*ora, proctolin was shown to increase amplitude of neurally-evoked contractions and cause a sustained increase in tonus, but could not evoke contractions in resting muscle (Bains & Downer, 1991). The magnitude of this response increased with increasing frequency of stimulation from 1 to 8Hz, suggesting that the peptide effects depend on the level of depolarization (Bains & Downer, 1991). Similar dependence on depolarization was also observed in locust oviducts (Noronha & Lange, 1997). Proctolin also increased tonic contractions in the muscles pre-treated with glutamate, indicating that the peptide acts directly on the muscle tissue (Bains & Downer, 1991). This coincides with the evidence that proctolin increases the frequency and amplitude of myogenic contractions of the oviducts (0.5-5nM; Lange et al., 1987) and foregut muscles (10^{-8} to 2 x 10^{-6} M: Gray et al., 1994) of *Locusta migratoria* and single muscle fibres of the marine isopod (*Idotea*) abdominal extensor muscles (Erxleben et al., 1995).

The enhancement of both nerve evoked (Bains & Downer, 1991) and myogenic contractions (Lange et al., 1987; Wegener & Nassel, 2000) by proctolin was abolished in calcium free saline and partially reduced in the presence of VGCC inhibitors, which suggests that proctolin exerts its effects via influx of extracellular Ca^{2+} through voltage gated Ca^{2+} channels. It has been proposed that the action of the proctolin on the locust oviducts (Lange et al., 1987) and the hindgut of a cockroach (Cook and Holman, 1985) involve non-voltage-gated Ca^{2+} channels.

In locust extensor tibialis muscle, iontophoretic application of proctolin at concentrations higher than 5 x 10^{-10} M depolarized the membrane (May et al., 1979), however, the stimulatory effects of proctolin on locust mandibular muscle (Bains and

Downer., 1991) and crayfish tonic flexor muscle (Bishop et al., 1987) were not accompanied by muscle depolarization. Tension recording and currrent-clamp recordings from single, isolated, abdominal extensor muscle fibres of the marine isopod *Idofea baltica* revealed that myostimulatory effects of proctolin are associated with it's ability to increase membrane input resistance (by approx.25%). The increase in input resistance was accomplished through reduction of the activity of non-voltage dependent K^+-permeable channels by approximately 63% (Erxleben, 1995).

The effect of proctolin on membrane input resistance and contraction amplitude was mimicked in the presence of membrane-permeable cAMP analog dibutyryladenosine 3', 5'-cyclic monophosphate (db-CAMP, 10^{-4} M), and inhibitor of the cAMP phosphodiesterase, 3-isobutyl- 1 -methyl-xanthine (IBMX, 10^{-4} M) (Erxleben, 1995) which suggest that cAMP and PKA (cAMP-dependent protein kinase) may be involved in mediating proctolin's myotropic effects. The action of proctolin is also likely to be mediated by the phospholipase C (PLC) pathway since proctolin increased the production of 1,4,5-inositoltrisphosphate (IP_3) from the foregut homogenates of the locust *S. gregaria* (Hinton & Osborne, 1995) and locust mandibular closer muscle (Baines et al.,1990). Proctolin-induced contractions in the hyperneural muscle of the cockroach *Periplaneta americana* were reduced by TMB-8, an non-selective blocker of intracellular Ca^{2+} release, thapsigargin, a blocker of the Ca^{2+} -ATPase of the sarcoplasmic reticulum, and ryanodine (10 mM), a ryanodine receptor antagonist which suggests that IP_3-independent Ca^{2+} release from the sarcoplasmic reticulum also plays a critical role in proctolin-induced contractions (Wegener & Nassel, 2000).

1.5.3 RF-amide peptides

Based on their C terminal sequence, insect RFamides are grouped into FMRFamide, HMRFamide, FLRFamide, RVRFamide and RLRFamide peptides (Nassel et al., 2002; Mercier et al., 2007; Orchard et al., 2001). The last two sub-groups, also referred to as neuropeptide F, are involved in reproduction, feeding and ecdysis behaviours (Mercier et al., 2001) and will not be dealt with in this review. In *Drosophila*, three groups of RFamide peptides, FMRFamides, myosuppressins (DMS; FLRFamides) and sulfakinins (DSK; HMRFamides) are encoded on separate genes, referred to as *dFMRFamide*, *Dms* and *Dsk*. These genes produce inactive precursor polypeptides that are further subjected to a series of posttranslational modifications to become bioactive peptides (Vanden Broeck, 2001; Nichols, 2003). The *Dms* gene encodes for one copy of myosuppressin (TDVDHVFLRFamide), and the *Dsk* gene encodes for three sulfakinins (DSK-0, DSK-I and DSK-II) (Vanden Broeck, 2001; Nichols, 2003). The *dFMRFamide* gene is a single copy gene that was first cloned by Nambu et al. (1988). It consists of two exons (106 and 1352 base pairs) and one intron (2500 base pairs) (Nambu et al., 1988; Schneider & Taghert, 1988). This gene encodes for 10 copies of FMRFamide-containing peptides (5 copies of DPKQDFMRFamide, 2 copies of TPAEDFMRFamide, and one copy each of SDNFMRFamide, SPKQDFMRFamide and PDNFMRFamide) and three other peptides (SVQDNFMHFamide, SAPQDFVRSamide and MDSNFIRFamide).

1.5.3.1 Distribution

RFamide-like immunoreactivity is present in central and peripheral nervous systems, as well as in neurosecretory cells in crustaceans (crayfish and lobster; Mercier

et al., 2001) and insects (Orchard et al., 2001; Nassel et al., 2002), suggesting that RFamides have neurotransmitter and neurohormonal functions. Approximately 44 out of 10 000 *Drosophila* neurons express the dFMRFamide gene, including interneurons, motor neurons and neurohaemal neurons (Schneider et al., 1993a,b). The expression of antiserum raised against FMRFamide showed that RFamides are present throughout the central nervous system (subesophageal ganglion, thoracic ganglia, the abdominal ganglia, medial and lateral protocerebrum, optic lobes and subesophageal ganglion) and the gastrointestinal system in all stages of *Drosophila* development (Nichols et al., 1999a). Using antisera raised to the specific N-terminus of DPKQDFMRFamide, SDNFMRFamide, and TPAEDFMRFamide peptides in a triple immunolabeling experiment, McCormic et al.(1999) showed that the expression patterns of these peptides encoded in the *Drosophila* FMRFamide gene are very distinct and they do not overlap. This suggested that the expression of FMRFamide peptides may be regulated at the posttranscriptional level. MALDI-TOF mass spectrometry profiling of the *Drosophila* larvae neurohaemal organs revealed the presence of DPKQDFMRFamide, TPAEDFMRFaamide, SDNFMRFamide, SPKQDFMRFamide, PDNFMRFamide, SAPQDFVRSamide and MDSNFIRFamide in the thoracic perisympathetic organs, a site of neurohaemal release from the thoracic and abdominal ganglia (Wegener et al., 2006). *Drosophila* myosuppressin (TDVDHVFLRFamide; DMS) was found in ring glands (Wegener et al., 2006), a structure that consists of three neurohemal organs, the corpora cardiaca, corpora allata and the prothoracic gland. The expression of *Drosophila*

FMRFamide-containing peptides and *Drosophila* myosuppressin in neurohemal organs further supports the idea that these peptides act as neurohormones.

1.5.3.2 Activity

Diverse physiological roles of RFamides are mostly related to their ability to modulate the contracture of different muscles (eg. heart, visceral and somatic muscles). This peptide-induced change in the muscle contractility can result from peptides acting on multiple targets, including central pattern generating circuits (CPGs), motor neurons, neuromuscular junctions and muscle fibres (Kravitz, 1988).

RFamides have been found to affect the heart rate in several animal species and can have both, inhibitory and excitatory effects. FLRFamides in lobster (TNRNFLRFamide; Worden et al., 1995) crayfish (DRNFLRFamide and NRNFLRFamide; Skerrett et al., 1995) and crab (NRNFLRFamide SDRNFLRFamide, and GYNRSFLRF ; Fort et al., 2007) increase the frequency and amplitude of muscle contractions in isolated hearts which may be partially caused by modulation of the impulse frequency in the cardiac motor neurons (Mercier et al., 2003). FMRFamide has stimulatory effect on the heart of *L. stagnalis*, since the peptide increases the frequency, amplitude and tonus of heart contractions in a dose dependent manner, with a threshold of approximately 100 pM and a maximum response occurring at 1μM (Willoughby et al. 1999b). *In vivo* recordings revealed that *Drosophila* FMRFamides and DMS affect heart rate as well, although the effects were inhibitory. SDNFMRFamide (1 μM and 10 μM injections) decreased the frequency of heart contractions to approximately 70-80% of basal heart rate in pupae and adult *Drosophila* flies within the first minute of peptide application, while DPKQDFMRFamide and

TPAEDFMRFamide (10 μM) had no effect on the contractility rate of pupal and adult heart muscle (Nichols et al., 1999b). In contrast, Johnson et al. (2000) reported that DPKQDFMRFamide reduced pupal heart rate in a dose-dependent manner with an EC_{50} at approximately 0.3 μM. PDNFMRFamide also reduced the heart rate with an EC_{50} of approximately 80 μM. Taken together, results suggest that FMRFamide containing peptides have inhibitory effects on heart contractions, however, the strength of the inhibitory effects differ among these peptides. The observed difference in the potency of the peptide's actions could be explained by the difference in their N-terminus sequences, which may be a critical factor for determining their binding affinities for the receptor (Table1). The other plausible reason for the difference in their effects is that they are binding to different receptors. Effects of DMS on pupal heart rate measured *in vivo* were more pronounced than those of FMRFamides (Merte & Nichols, 2002; Nichols, 2006). Within 30 s after the injection of 1 μM TDVDHVFLRFamide, the heart rate dramatically decreased to approximately 30 % of the basal level. The initial decrease in the frequency of muscle contractions was followed by a partial recovery to approximately 80 % of the basal heart rate that was sustained throughout the 10 min recording period (Nichols, 2006). A recent study by Nichols and colleagues (2009) reported that sulphated *Drosophila* sulfakinins (DSK I; FDDYGHMRFNH_2 and DSK II: GGDDQFDDYGHMRFNH_2) increased the frequency of larval, pupal, and adult heart contractions.

RFamides also affect the gut activity of crustaceans and insects. Lobster (TNRNFLRFamide and SDRNFLRFamide; 1 nM - 0.1 μM) and cockroach (pQDVDHVFLRFamide; 0.1-10 μM) FLRFamides enchanced spontaneous hindgut

contractions in crayfish, *P.clarkii* (Mercier at al., 1997). In contrast, DMS and FMRFamides had inhibitory effects on *Drosophila* gut contractility. The average rate of adult crop motility was reported to be approximately 47 contractions/ min (Kaminski et al., 2002). 1 μM (Kaminski et al., 2002) and 100 μM (Duttlinger et al., 2002) DMS completely stopped movement of the adult crop within 1 min of peptide application. Recovery of this effect was not observed during the 10 min recording period. The effect of FMRFamides on crop contractility was less dramatic. The maximal reduction to approximately 65% of basal crop contractility was observed within 2 minutes after 1 μM SDNFMRFamide application (Kaminski et al., 2002). Application of 100 μM TPAEDFMRFamide decreased the frequency of spontaneous crop contractions in the adult to 40% of the basal rate within 1 min (Duttlinger et al., 2002). Recovery of approximately 20% of crop movement was noted in 10 min. In contrast to the effects of TPAEDFMRFamide, 100 μM DPKQDFMRFamide and 100 μM of SDNFMRFamide had no effect on the frequency of spontaneous crop contractions (Duttlinger et al., 2002). Using an *in vivo* assay, Palmer et al (2007) recorded the frequency of adult crop contractions and larval anterior midgut in response to 100 μM DSK0 (NQKTMSFNH2). DSK0 decreased the frequency of the adult crop contraction rate to 34 % of the basal contraction rate, but it had no effect on larval gut contractions (Palmer et al., 2007).

In addition to cardiac and gut muscles, crustacean skeletal muscles are also targets for RFamide peptides. For example, TNRNFLRFamide potentiates nerve-evoked contractions in the opener muscle of lobster (Worden et al., 1995) and crayfish deep abdominal extensor muscles (Mercier et al., 1990). Similar excitatory effects of

FMRFamides on nerve-evoked contractions were obtained in *Drosophila* larval body wall muscles (Hewes et al., 1998). Five FMRFamides (DPKQDFMRFamide, TPAEDFMRFamide, SDNFMRFamide, SPKQDFMRFamide and PDNFMRFamide) enhanced nerve-evoked muscle tonus with the threshold of approximately 10 nM (EC_{50} :25 nM)(Hewes et al.,1998). Excitatory effects on nerve-evoked contractions appear to involve both, presynaptic and postsynaptic mechanisms. A presynaptic mechanism would involve peptide acting on the presynaptic terminal to enhance or facilitate the release of neurotransmitters that will, in turn, result in activation of a larger number of postsynaptic receptors, and, enhance the response of the muscle cell. DRNFLRFamide , for example, increases the number of quanta of transmitter released from nerve terminals onto deep abdominal extensor muscles without changing the amplitude of quantal synaptic currents (Skerrett et al., 1995). Hewes et al. (1998) showed that 0.1 µM DPKQDFMRFamide enhanced the amplitude of excitatory junctional currents (EJCs) by 50% in *Drosophila* muscle fibres. In addition to affecting EJCs, DPKQDFMRFamide (1 µM) significantly increased the amplitude of excitatory junctional potentials (EJPs) in larval muscles by approximately 20 % (Dunn & Mercier, 2005). This effect was neuron specific. EJPs elicited by stimulating one motor neuron (1b) were enhanced, but EJPs elicited by another neuron (1s) were not altered, even though EJPs occurred in the same muscle fibre. As with the effects of DPKQDFMRFamide on nerve-evoked muscle contractions, the effects on synaptic potentials were dose-dependent, with a threshold of approximately 10 nM and a maximal effect at approximately 1 µM (Dunn & Mercier, 2005).

The postsynaptic mechanism, on the other hand, assumes direct action of peptide on the muscle cell by binding to the receptor and activating a cascade of events that lead to an initiation of contractions. Postsynaptic actions of RFamides were confirmed in crustacean and insect models. In *P.clarkii*, for example, DRNFLRFamide increased the tonus of superficial extensor muscles of the crayfish abdomen, even in the presence of a spider toxin, that blocks glutamate receptors (Quigley & Mercier, 1997) and potentiated the contractions evoked by direct electrical stimulation of the deep abdominal extensor muscles after removing the synaptic terminals by digestion with collagenase (Mercier et al., 2003). In *Drosophila*, DPKQDFMRFamide induced larval body wall muscle contractions in the absence of nerve stimulation (Clark et al., 2008). This effect was noted even after glutamate receptors were desensitized with 7 mM glutamate, which suggests that DPKQDFMRFamide- induced muscle contractions do not result from increasing glutamate release from the presynaptic terminals (Clark et al., 2008) and thus, that peptide acts directly on muscle fibres to initiate muscle contraction. However, the possibility of peptide increasing spontaneous release of neurotransmitters other than glutamate has not been excluded. The intracellular mechanisms mediating the postsynaptic response are still largely unknown.

1.5.3.3 Mechanisms of action

There is compelling evidence that several second messengers may be involved in mediating the stimulatory effects of RFamides on synaptic output. FLRFamide and FMRFamide-induced enhancement in EJP amplitude at the crayfish (Noronha & Mercier, 1995) and *Drosophila* (Dunn & Mercier, 2005) neuromuscular junctions was abolished in

the presence of CaMKII (Ca^{2+}/calmodulin-dependent protein kinase, type 2) inhibitors. Protein kinase C (PKC) inhibitors potentiated the DRNFLRFamide –induced initial rise in EJP amplitude by approximately 15 % but also increased the rate of washout effects, suggesting that the PLC pathway plays a critical role in maintaining the stimulatory effect of peptide on neurotransmitter release, even after the peptide has been removed (Friedrich et al., 1998). PKA and PKG (cGMP-dependent protein kinase) inhibitors, (Rp-cAMPS and Rp-8-pCPT-cGMPS) reduced the effects of DRNFLRFamide on EPSP amplitude by approximately 75% and 40%, respectively. However, when both inhibitors were applied together, the combination completely abolished the ability of the peptide to enhance EPSP amplitude (Badhwar et al., 2006). Thus, presynaptic modulation appears to involve up to four protein kinase enzymes. In *Drosophila*, the mechanism by which DPKQDFMRFamide modulate synaptic output was shown to involve Ca^{2+} regulation. Using the calcium-sensitive calcium fluorophore, Oregon Green 488, Klose et al. (2010) showed that single stimuli applied to the nerve cause a transient rise of Ca^{2+} -induced fluorescence (Ca^{2+} peak amplitude) in individual tonic-like nerve terminals of axon 1b. The amplitude of these stimulus-evoked Ca^{2+} peaks was enhanced by 0.1 µM DPKQDFMRFamide, but the basal Ca^{2+} levels remained the same. Xestospongin (100 nM), an IP_3 receptor inhibitor and ryanodine (100 µM), which inhibits ryanodine receptors (RYR), abolished the ability of the peptide to increase the amplitude of stimulus-evoked Ca^{2+} peaks (Klose et al., 2010). The same result was obtained with IP_3 and RYR mutants (Klose et al., 2010). In addition, the peptide's effects on EJP amplitude were also abolished in these mutants (Dunn, T., 2003; Klose et al., 2010). Taken together, these data suggest that the peptide augments peak

Ca^{2+} levels in synaptic terminals via action on IP_3 receptors and ryanodine receptors associated with internal Ca^{2+} stores, and the resulting enhancement of CaMKII increases neurotransmitter release. The consequent increase in EJP amplitude would contribute to the observed enhancement of nerve-evoked contractions.

Although most of the identified RFamides exert either inhibitory or stimulatory actions on muscle contractions, not much is known about the mechanisms underlying their effects on excitation-contraction coupling.

FMRFamide increased the rates of $Ins(1,3,4)P_3$ production in the *L.stagnalis* heart tissue (1 μM FMRFamide; Willoughby et al., 1999b) and the tentacle retractor muscles of *Helix aspersa* (100 μM FMRFamide; Falconer et al., 1993). In contrast, pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH2 (pQDPFLRFamide), which was shown to relax the tentacle retractor muscles, did not affect $InsP_3$ levels (Falconer et al., 1993). Application of IP_3 to DMSO permeabalized isolated heart increased beat frequency (35±5% increase from controls) and muscle tonus (Willoughby et al., 1999b). Neomycin, a PLC inhibitor, reduced the cardioexcitatory effects of FMRFamide and the ability of FMRFamide to stimulate $Ins(1,4,5)P_3$ production. Together, these data provide evidence that IP_3 is an important factor in mediating the excitatory effects of FMRFamide peptide on muscle contractions.

However, FMRFamide also increased AC activity in heart tissue of *L.stagnalis* (Willoughby et al., 1999a). FMRFamide increased the rate of cyclic AMP production by approximately 3 fold from the control levels. The threshold concentration necessary to produce an increase in the rate of cyclic AMP production was approximately 10 nM, 10

fold higher than that observed with IP_3 production, which may suggest that cAMP is not the primary second messenger mediating peptide's effects. The cardioexcitatory effects of FMRFamide were mimicked by a membrane-permeable cyclic AMP analogue (8-bromo-cyclic AMP) and an AC activator (forskolin) (Willoughby et al., 1999a). Thus, the FMRFamide's myoexcitatory effects appear to be mediated by two different second messengers systems, cAMP and PLC-IP_3.

The effects of RFamides on muscle membrane properties are variable. In the crayfish deep abdominal extensor muscle, DRNFLRFamide and SDRNFLRFamide do not alter the input resistance, whereas TNRNFLRFamide and NRNFLRFamide increase input resistance by approximately 15 % (Mercier et al., 1990; Skerrett et al., 1995). The ability of peptide to increase input resistance suggests that it may be acting directly on the muscle tissue. Two-electrode voltage clamp recordings from the jumping muscle (m. extensor tibiae) of the locust *S. gregaria* revealed that the octopus peptide YGGFMRFamide, decreased the resting membrane conductance and the hyperpolarization-activated K^+ current by 38% and 46%, respectively (Walther, 1984). YGGFMRFamide also potentiated neurally-evoked muscle contractions by 2-3 fold (Walther, 1984). Hence, reducing the rectifying K^+ current may result in an increase in membrane excitability, which would account for the excitatory effects of this neuropeptide.

As seen with proctolin and CCAP-induced contractions, DPKQDFMRFamide-induced muscle contractions in *D. melanogaster* were also abolished in Ca^{2+} free saline (Clark et al., 2008). In addition, L-type calcium channel inhibitors, nifedipine and nicardipine,

completely abolished the ability of DPKQDFMRFamide to increase *Drosophila* body wall muscle tonus (Clark et al., 2008), suggesting that the peptide requires the influx of extracellular calcium via VGCC to induce muscle contractions in the absence of nerve stimulation. Hence, the model of action of DPKQDFMRFAmide is proposed (Fig 1A) in which the peptide acts on and activates the membrane bound receptor which in turn leads to an activation of VGCC, influx of calcium and activation of cascades of events leading to muscle contraction. However, the molecular mechanisms that bridge the gap between the binding of DPKQDFMRFamide to a receptor, including the identification of the receptor itself, and the influx of Ca^{2+} through L-type calcium channels are not known (Fig 2B).

1.6 Insect muscles

Larval locomotion is under the control of central pattern generators and consists of repeated peristaltic contraction waves that propagate from the head towards the posterior end of the larvae. This is accomplished by alternating between contracting the longitudinal muscles and oblique muscles in order to shorten and lengthen the body along its longitudinal axis, respectively (Peron et al., 2009).

All insect muscles are striated and each muscle fibre consists of the cell membrane (sarcolemma) and the inner sarcoplasm. In *Drosophila*, each larval hemisegment contains 30 supercontractile muscle fibres whose actin and myosin filaments are organized in a fashion similar to vertebrate striated muscles (Goldstein & Burdette, 1971). Each muscle is attached to an apodeme, which serves as a functional skeleton attaching the muscle fibre to the cuticle (Budnik and Gramates, 1999).

In vertebrate skeletal muscles, contraction is elicited by release of calcium from an intracellular store (the sarcoplasmic reticulum) through channels sensitive to ryanodine (Randall et al., 2002). The mechanisms involved in excitation-contraction coupling in *Drosophila* larvae are still under investigation. The trigger for contraction involves an increase in intracellular calcium concentration via influx of calcium through VGCC, which then leads to calcium-induced calcium release (CICR) from internal stores, presumably via opening of ryanodine receptor (RYR) channels in the membrane of the sarcoplasmic reticulum (SR) (Wang et al., 2003). Body wall contraction propagation (locomotion) was completely inhibited in *Drosophila* larvae that ingested ryanodine at a dose known to completely inactivate RYRs (100 mM) and was severely reduced in RyR mutant larvae (Sullivan et al., 2000), suggesting that RyRs indeed play a crucial role in larval muscle contraction. The effects of another protein found on the SR membrane that is involved in sequestering intracellular calcium, a sarcoplasmic/endoplasmic reticulum calcium pump (SERCA), on muscle contractions in *Drosophila* is largely unknown.

Similarly to vertebrate muscles, invertebrate muscles have a well developed T-tubule system (transverse tubular system) consisting of deep invagination of the muscle cell membrane into the muscle fibre, which enables the depolarization of the membrane to spread deep into the muscle (Rosomer and Stoffolano, 1994). In some invertebrate fibres, the influx of calcium through the cell membrane and T-tubules is sufficient for contraction (Gilly and Scheuer, 1984). In the skeletal muscles of vertebrates and muscles of some invertebrates, mostly molluscs and arthropods, the influx of calcium via cell membrane VGCC is coupled to the activation of RyR channels and release of calcium from

the SR (Loesser et al., 1992; Marx et al., 1998; Bers & Fill, 1998). This functional coupling is possible because of structures called "junctional feet", tetrameric arrays of RyRs connecting the gap between the T tubule and SR, where every other RyR is associated with a VGCC on the T tubule (Bers and Fill, 1998). However, the presence of junctional feet in *Drosophila* larval muscles is yet to be shown.

1.7 Objectives

In recent years, *Drosophila* has become a model of choice when investigating the modulatory effects of neuropeptides, for several reasons. First, all the motor neurons and muscle fibres in the larval body wall have been identified, making it possible to study synaptic interactions between known cell pairs. Secondly, the whole genome is sequenced and, with the continuous development of molecular and genetic techniques, an enormous number of genetic mutants and transgenic fly lines have become readily available to use for dissecting molecular particulates underlying physiological functions. With the development of UAS-GAL4 system, researchers are able to downregulate or upregulate the expression of specific genes in a temporal and tissue-specific manner.

Using *Drosophila* as a model system, the following three questions are addressed in this thesis (Fig 1B) :

1. Is the ability of DPKQDFMRFamide to induce muscle contraction mediated via a GPCR ?
2. Which secondary messenger systems are involved in mediating the peptide`s action on muscle?
3. Are the effects of DPKQDFMRFamide truly postsynaptic effects?

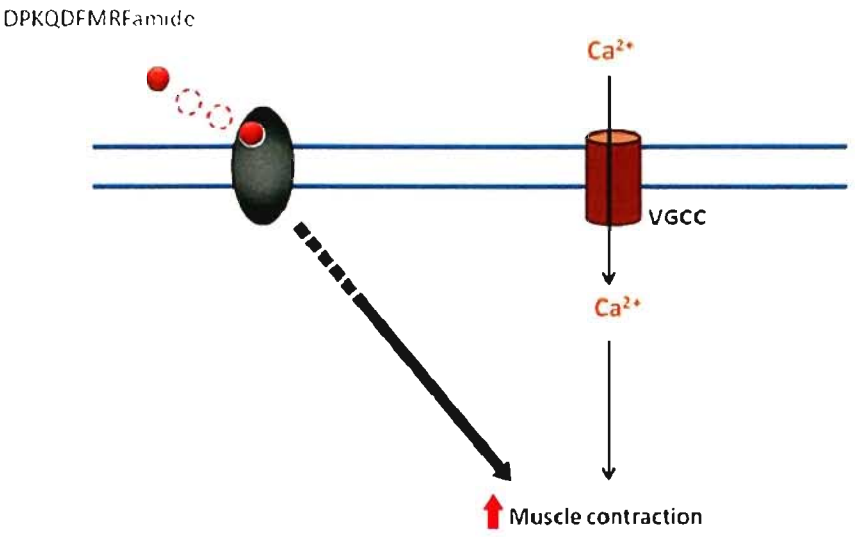
As described above, RFamides can act via two types of receptors, ligand-gated Na^+ ion channels and GPCRs. Four RFamide GPCRs were cloned and characterized in *Drosophila melanogaster*, but only one, FR (gene:CG2114), was found to be highly sensitive to dFMRFamide-containing peptides and less to other –RFamide peptides (Meeusen et al., 2002; Cazzamali & Grimmelikhuijzen, 2002). To demonstrate that DPKQDFMRFamide is acting via FR to elicit contractions in *Drosophila* larvae muscle, the expression of the FR gene (CG2114) was silenced using the UAS-GAL4 system. If the peptide acts via the FR, larvae in which the silencing of the FR gene was driven specifically in muscle tissue or ubiquitously should not respond to the peptide. If this peptide effect is exclusively postsynaptic, larvae in which the FR gene was silenced only in neurons should still respond to the peptide.

The evidence reviewed in the preceding sections indicates that several intracellular messenger signals mediate the effects of –RFamide peptides on muscle tissue, including cAMP and IP_3. A combination of pharmacological, biochemical and genetic approaches were used in the present study to investigate the possible involvement of these second messengers in mediating DPKQDFMRFamide's excitatory effects on the larval body wall muscle tissue. It was predicted that chemicals which inhibit the production or activation of cAMP and cGMP or its effects on kinase enzymes would abolish the peptide's effect if those second messengers were involved in mediating the peptide's effect. The functional role of cAMP and cGMP in mediating the peptide's effect was also confirmed by measuring their levels using an enzyme-linked immunosorbant assay. To establish if PLC- IP_3 secondary messenger pathway has a role

in mediating the peptide's effect, the effect of the peptide on muscle contraction were tested on larvae of several mutant and transgenic flies with disruptions in activity of proteins involved in PLC- IP_3 pathway.

Finally, although the CNS is removed, the motor neurons remained attached to the muscles when recording contractions from the whole body wall larval muscles. These motor neurons contain a number of different neurotransmitters, the most prominent one is glutamate, and the others, such as pituitary adenylate-cyclase activating polypeptide, octopamine, proctolin and insulin-like peptide (Peron et al., 2009, Budnik & Gramates 1999). Previously published evidence (Clark et al., 2008) showed that desensitizing glutamate receptors, and hence preventing the activation of glutamate receptors by glutamate released from presynaptic terminals, does not affect the ability of the peptide to induce contractions and increase muscle tension in *Drosophila* larval body wall muscles. This provides evidence that the peptide is not acting presynaptically to release glutamate, but it does not exclude the possibility that any of the other neurotransmitters are being released and further acting on larval muscle themselves. To give an answer to this question, I compared transduction pathways known to mediate the presynaptic effects to those that are mediating postsynaptic effects. The prediction was that if the peptide-induced increase in neurotransmitter release is responsible for the peptide's myogenic effects, then inhibiting the molecular mechanisms known to mediate the presynaptic effects would also abolish the peptide's effect on muscle contraction.

A)



B)

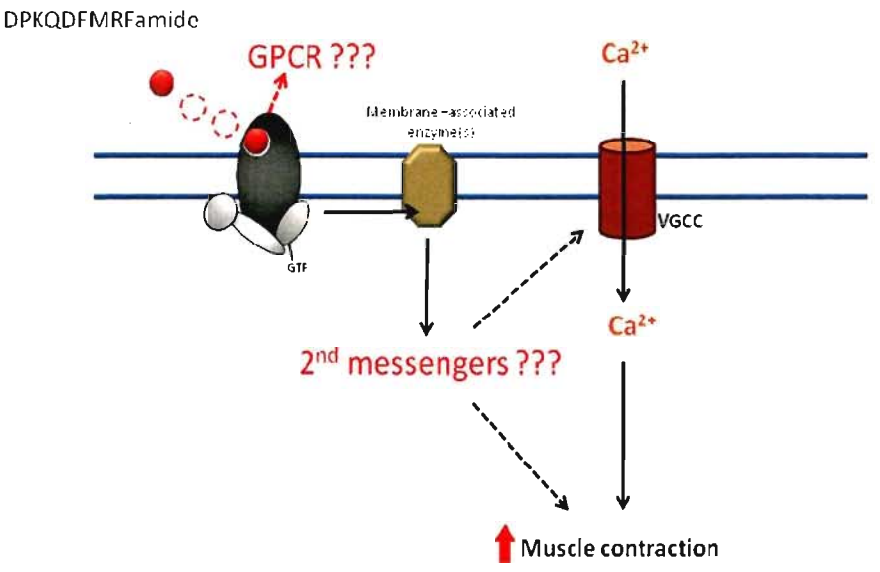


Figure 1. Model of DPKQDFMRFamde's action on body wall muscle contractions of *Drosophila melanogaster*. A) DPKQDFMRFamide induces phasic contractions and increases body wall muscle tonus (Clark et al., 2008). This effect depends on the activation of voltage gated calcium channels (VGCC). B) DPKQDFMRFAmide exerts its myotropic effects, presumably, by activating a muscle membrane receptor. This study aims to examine if the peptide acts through a GPCR encoded on the *Drosophila* FR gene (CG2114) and whether it requires activation of second messengers, more specifically, it examines the involvement of cAMP, cGMP, IP_3 and CaMKII.

2. METHODS

2.1 Fly stocks

Canton S (CS) flies, obtained from Bloomington *Drosophila* Stock Centre (BDSC) were used for the experiments unless otherwise indicated.

The IP_3 receptor mutant used was Itp-r83A^{05616} {P{PZ}Itp-r83A^{05616} Nmdar1^{05616} ry^{506}/TM3, ry^{RK}Sb^{1}Ser^{1}} (BDSC, donated by Berkeley *Drosophila* Genome Project). This mutation had been generated by inserting a single transposable element in the 3rd chromosome to disrupt the IP_3 receptor encoding gene, *itp-r83A* (Spradling et al., 1999). Since this mutation was balanced over a Sb^{1}Ser^{1} dominantly marked balancer chromosome, a genetic cross scheme was designed to remove this balancer. First, a cross between the IP_3 mutant receptor line and the w*; Sb^{1}/TM3, P{ActGFP}JMR2, Ser^{1} balancer line (BDSC) was constructed to obtain progeny with the P element insertion balanced over TM3, P{ActGFP}JMR2, Ser^{1}. Then, the selected progeny line was further crossed with w ^{1118} flies (BDSC) to remove the GFP, Ser^{1} marked balancer chromosome (Fig.2).

Four different fly lines with mutations in two genes encoding for PLCβ were used. These included three mutants of the *norpA* (*no receptor potential*) gene, which were: w* norpA^{33}, w*norpA^{36} and norpA^{7} (BDSC). OregonR was the wild type strain used as a control for *norpA* gene mutants. In addition, the *Plc21C* gene mutation line (y^{1}w^{1118}; PBac{5HPw^{+}}Plc21C^{A246}) was used with its corresponding control line (y, w), both of which were acquired from BDSC.

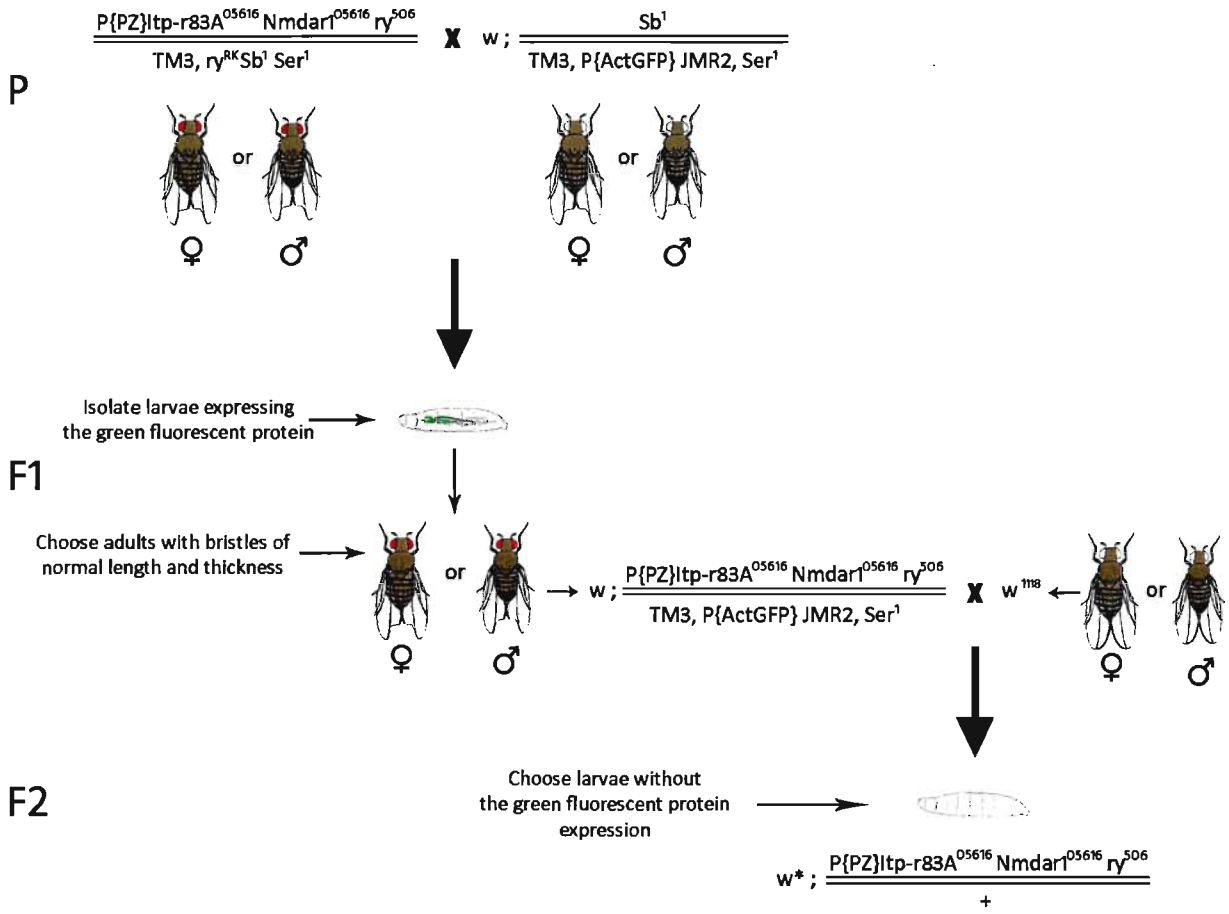


Fig.2. Schematic diagram of the genetic cross used to remove the balancer chromosome from the IP_3 receptor mutants and to obtain 3rd-instar larvae heterozygous for the IP_3 receptor mutation (w; P{PZ}Itp-r83A^{05616} Nmdar1^{05616} ry^{506}/ +) that could be further tested in the muscle contraction assay. Male and virgin female flies heterozygous for the P element insertion (P{PZ}Itp-r83A^{05616}) and the Sb, Ser marked balancer were crossed to either virgin females or males heterozygous for Sb and the green fluorescent protein (GFP), Ser marked balancer, respectively. From the progeny of this cross, only 3rd instar larvae expressing GFP were individually isolated and allowed to reach the adult stage. From these, only male and virgin female adult flies with normal bristles (non Sb expressing) were selected (w; P{PZ}Itp-r83A^{05616} Nmdar1^{05616} ry^{506}/ TM3, P{ActGFP}JMR2, Ser^{1}) for the next cross. Finally, to remove the GFP, Ser marked balancer chromosome, male or virgin female flies from F1 were crossed with flies of the opposite sex, carrying wild type 3rd chromosome and w ^{1118} mutation on the X chromosome. 3rd-instar larvae of the F2 progeny lacking GFP expression (w; P{PZ}Itp-r83A^{05616} Nmdar1^{05616} ry^{506}/ +) and the control line (w ^{1118}) were assayed for DPKQDFMRFamide-induced muscle contractions. ry, rosy; Sb, Stubble; Ser, Serrate.

To investigate the role of CaMKII an ala 1 {w P[w+ ala]} transgenic line was used. This line had been constructed by inserting a synthetically generated alanine inhibitory peptide gene on the first chromosome under the control of a heat shock promoter (Griffith et al, 1993). The gene for the synthetic alanine inhibitory peptide was constructed based on the sequences of the rat α CaM kinase autoregulatory domain (Griffith et al., 1993). The UAS-ala line contains an alanine inhibitory peptide gene inserted downstream of UAS and was used as a control for ala1 line. Both transgenic lines were generous gifts from Dr Leslie Grifith (Brandeis University, Mass).

A transgenic line containing FMRFamide receptor inverted repeat (FR-*IR*) downstream from the upstream activating sequence (UAS) was obtained from the Vienna *Drosophila* RNAi Center (VDRC #9594).The generation of this line (UAS-*FR* RNAi) was described by Dietzl et al. (2007). Briefly, a FMRFamide receptor gene fragment was cloned as a 301bp long inverted repeat (IR) in antisense-sense orientation into a modified pUAST vector, pMF3 with multiple UAS sites. This cloned construct was then inserted on the second chromosome of an isogenic w^{1118} host, generating a homozygous viable UAS-*FR* RNAi line.

The following driver lines were used to express the RNAi for the FR: *elav-GAL4* (BDSC) and *24B*-GAL4 (BDSC) and *tubP*-GAL4 (BDSC). *elav*-GAL4 is a homozygous and viable transgenic line with the P{GAL4-elav.L} construct inserted at the third chromosome. The neuron-specific promoter from the gene *elav* drives the expression in all postmitotic neurons starting at stage 12 of embryonic development (Luo et al, 1994, Sink et al., 2001). In this study, *elav*-GAL4 was used for pan-neuronal expression of the

UAS-FR-IR transgene. *24B*-GAL4 is a homozygous enchancer trap line that expresses GAL4 in mesoderm as early as stage 11 of embryonic development (Luo et al., 1994, Brand and Perrimon, 1993) and in all larval somatic muscles (Schuster et al.,1996). *tubP*-GAL4 is an insert on the third chromosome that is balanced over TM3, *Sb* and allows for ubiquitous expression of Gal4 (Lee and Luo, 1999).

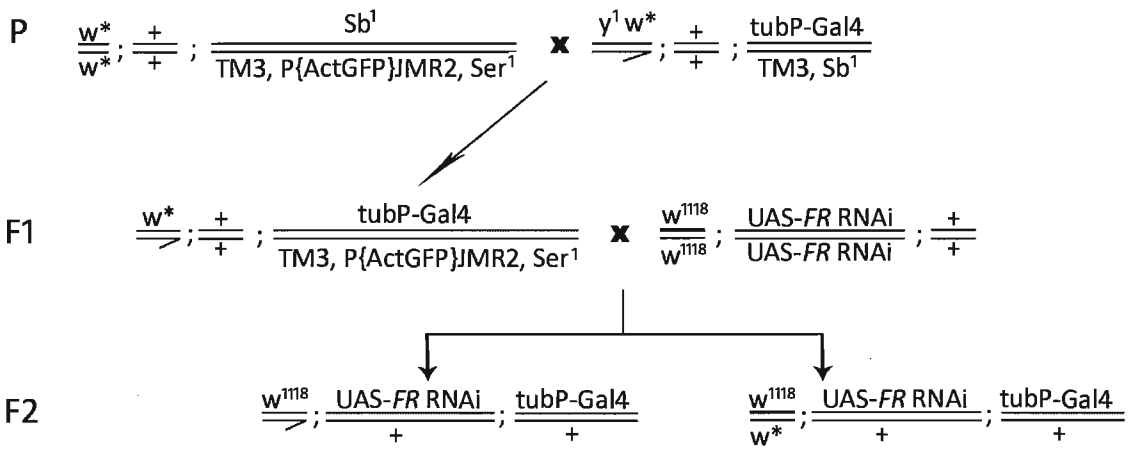
All flies were raised on a cornmeal-based medium (Boreal Laboratories Ltd., St. Catharines, Ontario, Canada), supplemented with dry yeast, at 21 °C on a 12:12 light–dark cycle.

2.2 Genetics

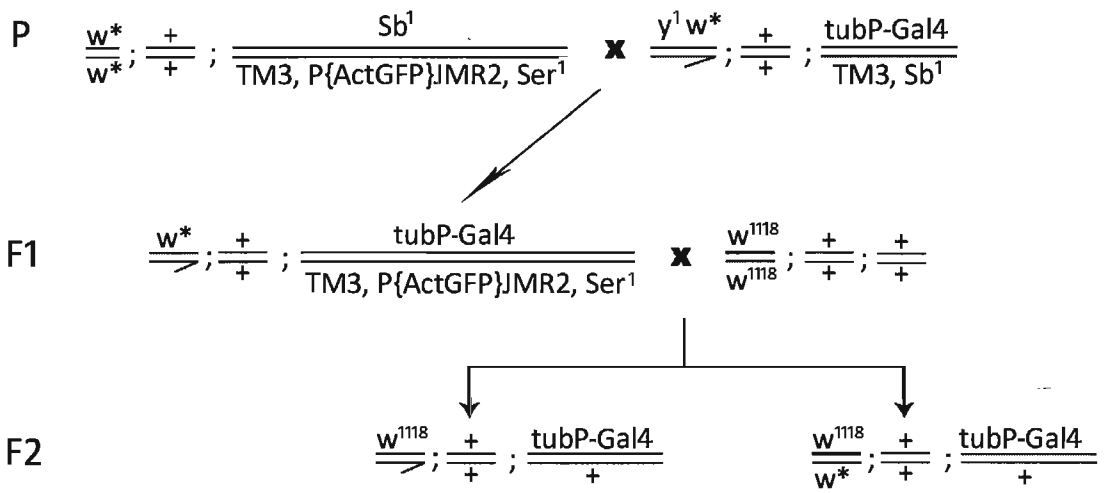
Tissue-specific expression of the UAS-*FR* IR construct was driven using the UAS/GAL4 system as described by Brand and Perrimon (1993). To express the UAS-*FR* IR construct ubiquitously, homozygous UAS-*FR* RNAi virgin females were crossed to *tubP*-GAL4 males that were previously balanced over TM3, P{ActGFP}JMR2, Ser^1 (Fig 3A). In addition, *tubP*-GAL4 males balanced over TM3, P{ActGFP}JMR2, Ser^1 were crossed with w^{1118} virgin females to remove the balancer chromosome and generate a heterozygous control line (Fig 3B).Expression of the UAS-*FR* IR construct in muscle and neuronal tissues was accomplished by crossing homozygous UAS-*FR* RNAi virgin females to homozygous *24B*-GAL4 and *elav*-GAL4males, respectively. Heterozygous larvae of the F1 generation were used for the experiments (Fig. 4A, 5A). To generate appropriate heterozygous Gal4 controls, homozygous elav-*GAL4* and *24B-GAL4* males were crossed to w^{1118} virgin female flies (Fig. 4B, 5B). To generate appropriate control larvae with non-activated UAS-*FR* construct, homozygous UAS-FR RNAi virgin females were mated

Fig. 3. Schematic diagram representing crosses designed to generate larvae with ubiquitous knockdown/reduction of FR expression (A) and their respective Gal4 control line (B). First, to be able to differentiate between 3rd instar larvae of different genotypes, I took advantage of a GFP marked balancer. Larvae carrying the GFP marked balancer express GFP markedly in the salivary duct, the copper cells, the proventriculus and the visceral musculature of the midgut (Reichhart and Ferrandon, 1998) and are easily recognizable under a fluorescent dissecting microscope. Hence, I replaced the Sb marked balancer with the GFP, Ser marked balancer. This was accomplished by crossing male flies heterozygous for Sb and the green fluorescent protein (GFP), Ser marked balancer (w^{1118}; + ; Sb^1/TM3, P{ActGFP}JMR2, Ser^1)with virgin females carrying one copy of the tubP-Gal4 insert balanced over the Sb marked balancer (w^{1118}; +; tubP-Gal4/ TM3, Sb^1) (A,B). From the progeny of this cross (F1), only 3rd instar larvae expressing GFP were individually isolated and allowed to reach the adult stage (A,B) . From these, only male and virgin female adult flies with normal bristles (non Sb expressing) were selected to obtain flies with w^{1118}; tubP-Gal4/TM3, P{ActGFP}JMR2, Ser^1 ; + genotype for the next cross. Next, male flies selected from the F1 generation (w^{1118}; tubP-Gal4/TM3, P{ActGFP}JMR2, Ser^1 ; +) were crossed with either A) virgin females homozygous for the UAS-FR inverted repeat construct on the second chromosome (w^{1118}; UAS-*FR* RNAi ;+) to obtain progeny (F2) expressing a single copy of the UAS-*FR* inverted repeat construct under the control of a single copy of tubP-Gal4 (w^{1118}; UAS-*FR* RNAi/+ ; tubP-Gal4/+) or B) with wild type virgin females (w^{1118}; + ;+) to generate larval progeny (F2) with one copy of the tubP-Gal4 insert on the third chromosome (w^{1118}; tubP-Gal4/+) . ry, rosy; Sb, Stubble; Ser, Serrate.

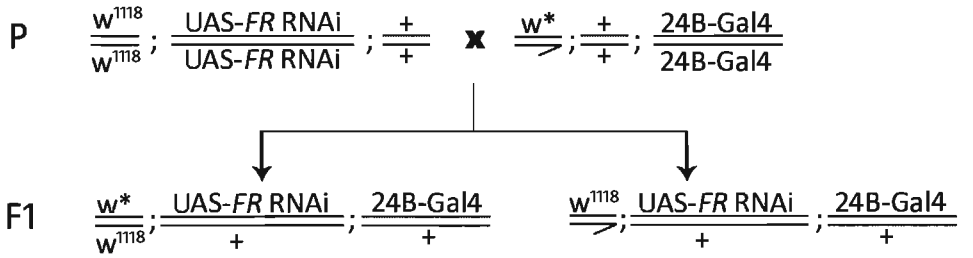
A)


P
w*/w* ; +/+ ; Sb1/TM3, P{ActGFP}JMR2, Ser1 x y1 w*/Y ; +/+ ; tubP-Gal4/TM3, Sb1
F1
w*/Y ; +/+ ; tubP-Gal4/TM3, P{ActGFP}JMR2, Ser1 x w1118/w1118 ; UAS-FR RNAi/UAS-FR RNAi ; +/+
F2
w1118/Y ; UAS-FR RNAi/+ ; tubP-Gal4/+
w1118/w* ; UAS-FR RNAi/+ ; tubP-Gal4/+

B)


P
w*/w* ; +/+ ; Sb1/TM3, P{ActGFP}JMR2, Ser1 x y1 w*/Y ; +/+ ; tubP-Gal4/TM3, Sb1
F1
w*/Y ; +/+ ; tubP-Gal4/TM3, P{ActGFP}JMR2, Ser1 x w1118/w1118 ; +/+ ; +/+
F2
w1118/Y ; +/+ ; tubP-Gal4/+
w1118/w* ; +/+ ; tubP-Gal4/+

A)



B)

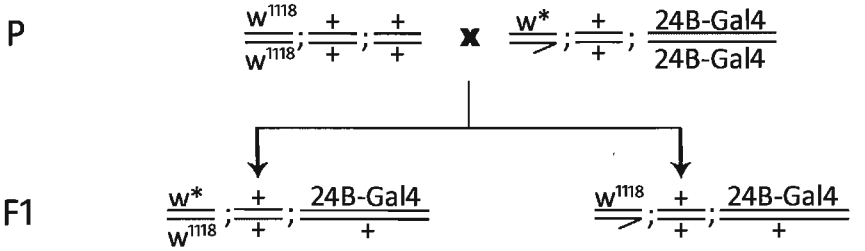
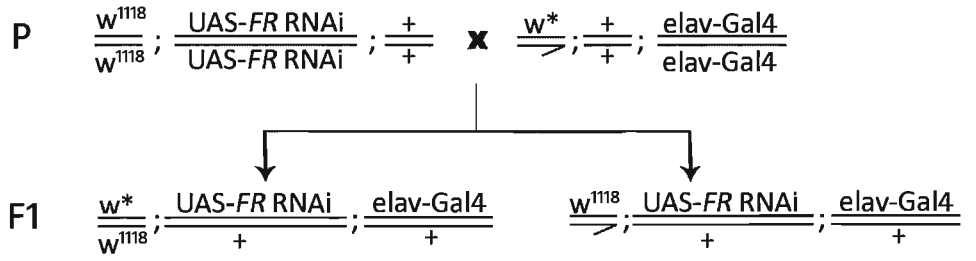


Fig.4. Schematic diagram representing crosses designed to generate larvae with a muscle-specific knockdown/reduced expression of FR (A) and an appropriate Gal4 control line (B). A) Virgin females homozygous for the UAS-*FR* inverted repeat construct on the second chromosome (w^{1118}; UAS-*FR* RNAi ; +) were crossed with males homozygous for the 24B-Gal4 insert on the third chromosome (w^{1118}; +; 24B-Gal4) to obtain progeny (F1) expressing a single copy of the UAS-*FR* inverted repeat construct under the control of a single copy of 24B-Gal4 (w^{1118}; UAS-*FR* RNAi /+ ; 24B-Gal4/+). B) In parallel, males homozygous for the 24B-Gal4 insert on the third chromosome (w^{1118}; +; 24B-Gal4) were crossed with wild type virgin females (w^{1118}; + ;+) to generate larval progeny with one copy of the 24B-Gal4 insert on the third chromosome (w^{1118}; +; 24B-Gal4/+).

A)



B)

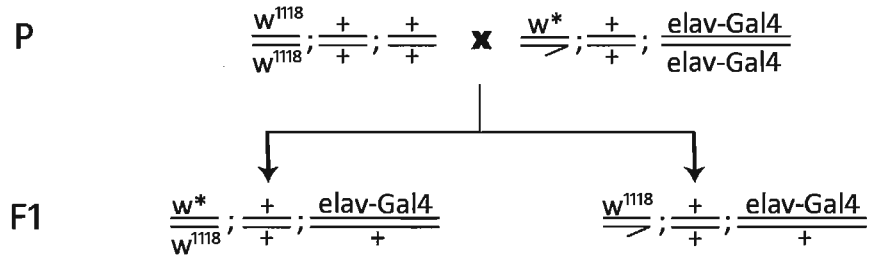


Fig. 5. Schematic diagram representing crosses designed to generate larvae with a neuron specific knockdown/reduced expression of FR (A) and an appropriate Gal4 – control line (B). A) Virgin females homozygous for the UAS-*FR* inverted repeat construct on the second chromosome (w^{1118} ; UAS-*FR* RNAi ; +)were crossed with males homozygous for the elav-Gal4 insert on the third chromosome (w^{1118} ; +; elav-Gal4) to obtain the progeny (F1) expressing of a single copy of the UAS-*FR* inverted repeat construct under the control of a single copy of elav-Gal4 (w^{1118} ; UAS-*FR* RNAi/+ ; elav-Gal4/+). B) In parallel, males homozygous for the elav-Gal4 insert on the third chromosome (w^{1118} ; +; elav-Gal4) were crossed with wild type virgin females (w^{1118}; + ;+) to generate larval progeny with one copy of the elav-Gal4 insert on the third chromosome (w^{1118}; +; elav-Gal4/+).

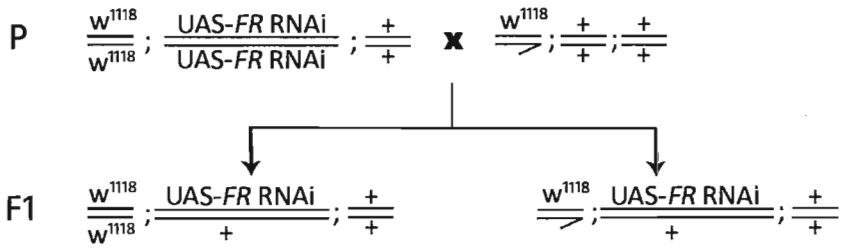


Fig.6. Schematic diagram of the cross designed to generate a control fly line with non-activated UAS-FR inverted repeat construct expression (w^{1118}; UAS-*FR* RNAi/+). Virgin female flies homozygous for the UAS-FR construct (w^{1118}; UAS-*FR* RNAi; +) were crossed with male wild type flies (w^{1118}; + ;+) to generate a fly line heterozygous for the UAS-*FR* construct on the second chromosome. The F1 generation 3rd instar larvae were tested in the muscle contraction assay.

to w^{1118} virgin female flies (Fig 6). All crosses were kept at 27°C on a 12:12 light-dark cycle.

2.3 Preparation

Wandering stage third-instar larvae were used for all experiments. They were collected from the sides of their culture vials immediately before dissections were made. All dissections were made at room temperature (~21 °C) in modified hemolymph-like (HL6) *Drosophila* physiological saline (Macleod et al, 2002). The saline contained (in mM): 23.7 NaCl, 24.8 KCl, 0.5 $CaCl_2$, 15 $MgCl_2$, 10 $NaHCO_3$, 80 trehalose, 20 Isethionic acid, 5.7 L-alanine, 2.0 L-arginine, 14.5 glycine; 11.0 L-histidine; 1.7 Lmethionine; 13.0 L-proline; 2.3 L-serine; 2.5 L-threonine; 1.4 L-tyrosine; 1.0 L-valine and 5 BES, pH 7.2. The saline was stored at -20 °C in 200 mL aliquots.

The larvae, oriented dorsal side up, were pinned down at the anterior and posterior ends to a Sylgard (Dow Corning Corporation, Midland, ML) dish containing saline. The larvae were stretched slightly longitudinally, and a mid-dorsal cut was made along the length of the animal (Fig 7, top). The entrails were removed, and the segmental nerves were cut near the ventral ganglion, which was subsequently removed along with the brain, as previously described by Jan and Jan (1976). Subsequently, the preparations were used for either the muscle contraction recording or enzyme immunoassay.

2.4 Muscle contraction recordings

After the dissection, the pin in the anterior end of the larva was removed and simultaneously the anterior tip of the larva was hooked to a Grass FT03 tension

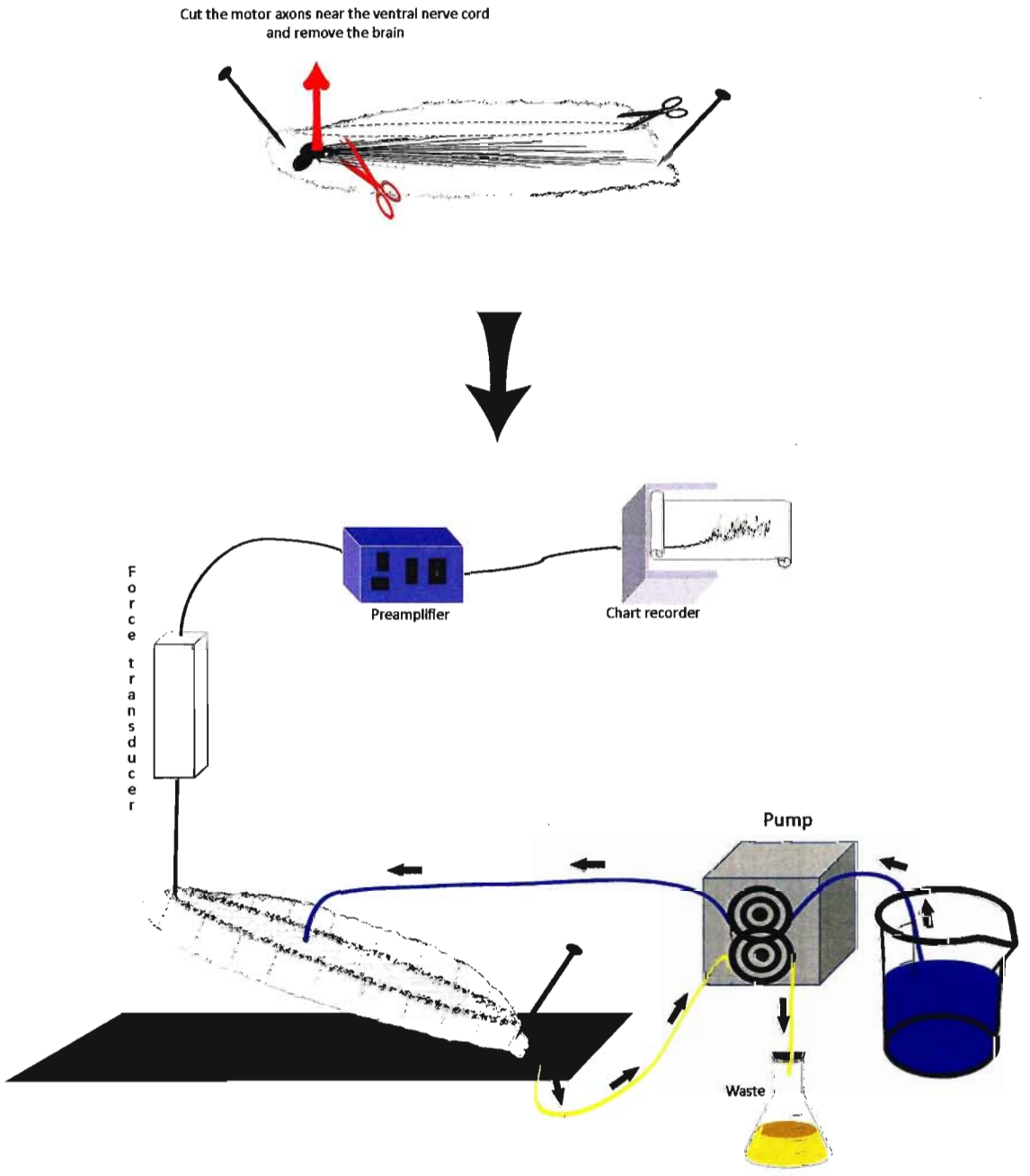


Fig 7. Schematic diagram of the *Drosophila* larval dissection and the experimental setup used to record body wall muscle contractions.

transducer (Grass Instruments, Quincy, MA) as previously described (Clark et al.,2008). Briefly, an L-shape bent insect pin was glued to a 54 mm metal rod which was attached to the force transducer at an angle that was perpendicular to the spring. The bent insect pin was hooked to the anterior end of the animal in such a way that contractions of the longitudinal muscles were parallel to the movement of the force transducer's spring. The larval body wall muscles were stretched slightly longitudinally by pulling the body in a dorsal and anterior direction until the larva was situated at an angle approximately 45 ° from horizontal (Fig 7, bottom). Contractions were amplified using a MOD CP 122A amplifier (Grass Telefactor, W. Warwick, RI) and were recorded on an Omniscribe chart recorder (Austin, Texas, USA). Calibration was performed using a pin of known mass and the following conversion, 1 mg= 0.009807 mN.

The recording dish had a volume of approximately 0.2-0.4 ml and was continuously perfused at a rate of 0.7 ml/min. As in previous experiments (Clark et al, 2008), applications of DPKQDFMRFamide caused irregular, rapid contractions, superimposed on a slow contraction lasting several minutes. Because the fast contractions were highly variable in occurrence and magnitude, the effect of the peptide was quantified (as in Clark et al, 2008) as the magnitude of the slow contractions, which corresponded to an increase in muscle tonus. To investigate the effect of peptide on muscle tonus in third instar larvae from the F1 generation of the various crosses, the peptide was applied for 5 min, and the maximal tonus was compared to the tonus during a 5-min pre-peptide period (basal tonus) to obtain the change in tonus. The peptide was washed out with saline for approximately 15 min, after which 300mM KCl

was applied for 5 min to ensure that the preparation was able to contract. The results are reported as means ± standard errors of mean (SEM).

The effects of DPKQDFMRFamide and different pharmacological agents were examined by exchanging experimental solutions. Excess fluid was removed by continuous suction. To investigate the effects of different pharmacological agents on the DPKQDFMRFamide-induced tonus change, the following procedure was followed. The peptide was applied for 5 min and washed out for approximately 15 min, which was followed by 10 min (20 min for KN-93) of perfusion with saline containing the chemical of interest and then 5 min of perfusion with saline containing the chemical and peptide.

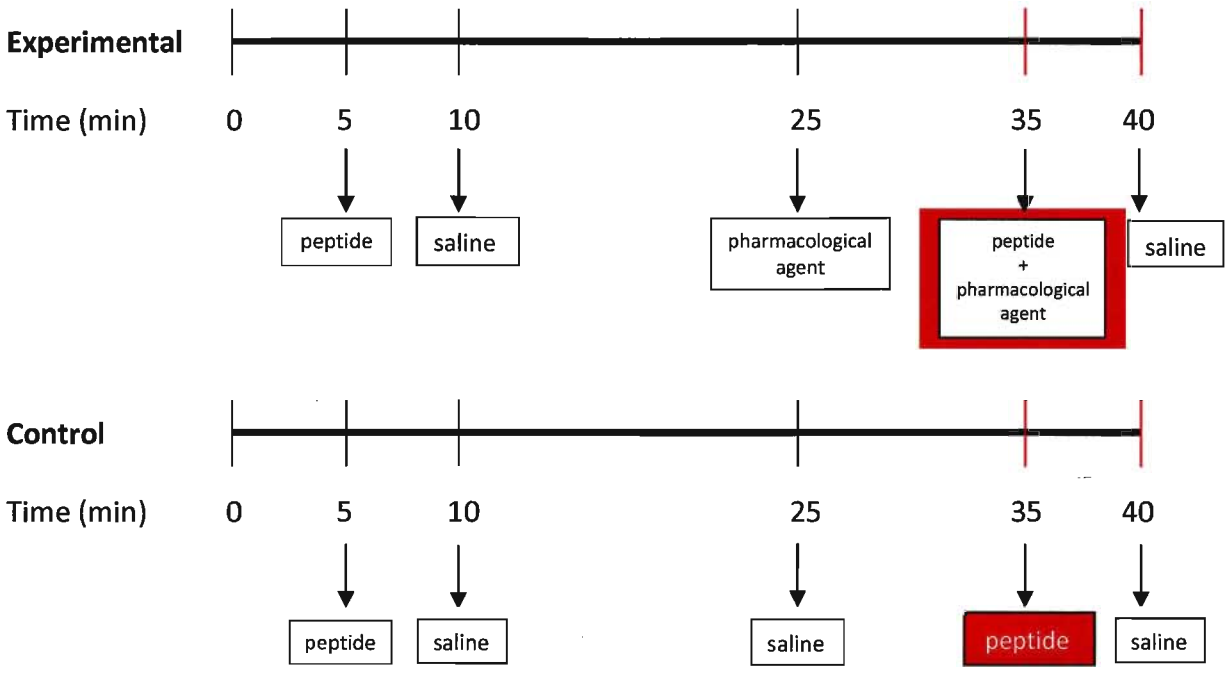


Fig 8. Schematic diagram demonstrating the time line and the order of solution applications during the muscle contraction recordings.

In the control trials the same perfusion procedure was followed, but the pharmacological agents were omitted from the solutions (Fig. 8). When DMSO was used to dissolve the chemical of interest, the DMSO concentration was consistent throughout each trial, and it was matched in control trials. To establish the effects of applied chemicals, the maximum tonus change during the 5 min peptide + chemical application period in the experimental trials was compared to the maximum tonus change within the second 5 min peptide application period in the control trials.

2.5 Heat-shock

Ala 1 and UAS-ala flies were placed in empty glass vials and placed in a dry incubator for 1 hour at 37 °C. After the heat-shock, flies were allowed to recover at room temperature (21°C) for approximately two hours before muscle contractions were recorded.

2.6 cAMP and cGMP enzyme immunoassays.

Dissected preparations of larval body wall muscles were placed in a Petri dish containing cold saline from which pools of 2-9 larval preparations were transferred into 100 µl of desired incubation solution. Individual pools of tissues to be tested for cAMP content were incubated separately in either (a) saline, (b) a non-selective phosphodiesterase inhibitor 5×10^{-4} M 1 3-isobutyl-1-methylxanthine (IBMX), (c) 5×10^{-4} M IBMX containing either DPKQDFMRFamide at concentrations ranging from 10^{-8} to 10^{-6} M or 5×10^{-5} forskolin (an adenylate cyclase activator) (d) 5×10^{-4} M IBMX containing 5×10^{-5} forskolin or (e) 5×10^{-5} M forskolin alone. Preparations to be assayed for cGMP were incubated in either (a) saline alone, (b) saline containing 5×10^{-4} M IBMX or (c)

saline containing 5 x 10^{-4} M IBMX with concentrations of DPKQDFMRFamide ranging from 10^{-8} to 10^{-6} M. All incubation solutions contained 0.25 % DMSO with the exception of solutions containing forskolin, which had 0.65% as the final DMSO concentration. After 10 minutes of incubation at room temperature, each larval pool was placed into 100 µl of 0.1 M hydrochloric acid (HCl) for 5 min to inhibit endogenous phosphodiesterase activity. To halt the chemical reactions, the larvae were placed onto a steel plate cooled over dry ice, which resulted in immediate tissue freezing. Frozen tissue was homogenized in 500 µl of 0.1 M HCl and then centrifuged for 12 min at 11,000 x g. The supernatant was transferred into fresh 1.5 ml Eppendorf tubes and stored at -80 °C until ready to assay for cAMP or cGMP content. The pellet was further submitted to a protein determination assay.

Levels of cAMP or cGMP were determined in duplicates from supernatant (100 µl) following the acetylated protocol for a direct cAMP enzyme immunoassay kit or overnight acetylated version of a direct cGMP enzyme immunoassay kit (Assay Designs, Ann Arbor, USA). Standards and supernatants were acetylated by adding 10 µl of acetylating reagent for each 200 µl of supernatant or standard. The acetylating reagent was prepared as a 2:1 mixture of triethylamine (99.5%) and acetic anhydride (98%) supplied in the kit. The sample and standard wells were prepared by adding neutralizing agent, cAMP (or cGMP) conjugate and a rabbit polyclonal antibody to cAMP (cGMP) into wells coated with goat antibody specific to rabbit IgG. Supernatant or standards were added to the wells, and each plate tested contained a set of blank controls and non-specific binding controls. The cAMP plates were incubated on a plate shaker (300rpm) at

room temperature for 2 hours. cGMP plates were incubated overnight at 4° C. After the appropriate incubation period, plates were washed a total of 3 times with a wash buffer using a Bio-Tek EL x 50™ automated strip washer (BioTek, Vermont, USA). After the final wash, a p-nitrophenyl phoshate buffer solution was added to each well, after which the plates were incubated for one hour at room temperature. The reaction was stopped by adding 50 µl of a trisodium phosphate solution (10%) to each well. The optical densities were read at 405 nm with a Bio-Tek Synergy™ HT microplate reader (BioTek, Vermont, USA), and the concentrations of cAMP (pmol/ml) or cGMP (pmol/ml) were estimated using a 4 parameter logistic curve-fitting program provided in the Bio-Tek KC4™ Software. The sensitivities of the acetylated version of the cAMP and cGMP assays were 0.037 pmol/ml and 25 pmol/ml, respectively.

Each pellet was dissolved in 100 µl of 1M NaOH and placed for 2 hours in a hot water bath (40 °C). Protein content was measured from 10-15 µl of solubilized pellet with a BioRad Protein Assay kit (based on method of Bradford, 1976) using bovine serum albumin as a protein standard. Absorbances were read at 595 nm using a spectronic 20 (Bausch and Lomb incorporated, USA).

To account for variations in the number and sizes of larval body wall preparations, the final results are expressed as pmol per mg of protein.

2.7 Chemicals and Peptide

The *Drosophila* peptide, DPKQDFMRFamide, was synthesized by Cell Essentials (Boston, Mass.) and was 98% pure as determined by reverse-phase High Performance Liquid Chromatography (HPLC). Peptide was stored at -20 °C and was dissolved in saline

to yield a 10 mM stock solution. 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Sigma-Aldrich (Oakville, ON., Canada). 5 mM IBMX stock solution was made in 0.5 % DMSO containing saline and kept at -20 °C. The DMSO concentration in the final IBMX solution used for physiological recordings did not exceed 0.05%. Forskolin, adenosine 3',5'-cyclic monophosphorothioate, Rp-Isomer, triethylammonium salt (Rp-cAMPS), guanosine 3',5'-cyclic monophosphorothioate, 8-(4-Chlorophenylthio)-, Rp-Isomer, triethylammonium salt (Rp-8-pCPT-cGMPS) and KN-93 were obtained from Calbiochem. Forskolin and KN-93 were dissolved in 100 % DMSO and stored at 4 °C as stock solutions (12 and 10 mM, respectively), which were subsequently diluted in saline to yield the desired drug concentration with final DMSO concentrations of 0.4 % and 0.1 %, respectively. 5 µmol of Rp-cAMPS and 1 µmol of Rp-8-pCPT-cGMPS were stored at -20 °C until ready to dilute in saline to yield the desired final concentration. All experimental solutions were made fresh on the day of testing.

2.8 Analysis

Both one way ANOVA and t-test for independent samples, unless otherwise stated, were used where appropriate to determine statistical significance, and $p<0.05$ was used for acceptance of statistical significance. All data were expressed as means ± standard error of the mean (SEM). The number of preparations is indicated in the parentheses on the graphs (n).

3. RESULTS

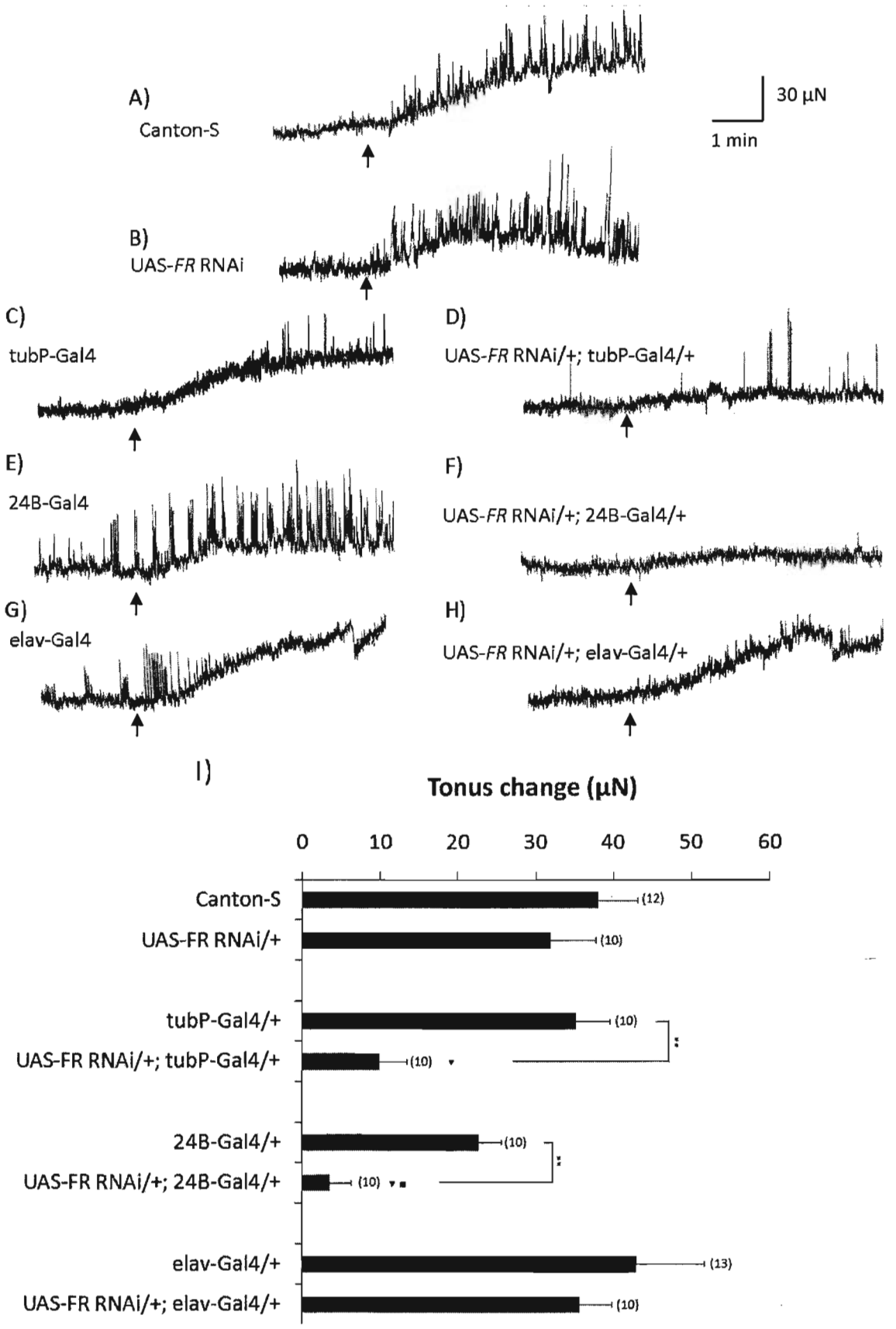
3.1 FMRFamide G-protein coupled receptor (FR) mediates the stimulatory effect of DPKQDFMRFamide on *Drosophila* larval body wall muscles

To investigate whether the GPCR encoded by the *Drosophila* FR gene (CG-2114) plays a role in mediating the effects of DPKQDFMRFamide on muscle tonus, a combination of genetic tools known to induce tissue-specific silencing of gene expression was used to alter the function of this gene in *Drosophila* larvae. One of the most prominent mechanisms used for gene silencing is RNA interference (RNAi). In this post transcriptional gene silencing system, a double-stranded RNA targets other homologous RNAs which are subsequently destroyed by the RNA-induced silencing complex (RISC), resulting in the reduction of the targeted gene expression. Several genetic methods have been developed to introduce double-stranded RNAs into different organisms, including the transfection of *Drosophila* cells with an inverted repeat (IR) transgene construct (Ashburner et al., 2005). An IR transgene construct contains a gene sequence that is complementary to a portion of the target gene and is transcribed into a hairpin RNA (one-stranded RNA folded on itself) that is able to target the transcripts of the same target gene. Transcription of an inverted repeat (IR) transgene construct under the control of UAS/GAL4 system (Brand and Perrimon, 1993) has been successfully used to induce tissue-specific posttranscriptional silencing of gene expression in *Drosophila* (Fortier and Belote, 2000, Kennerdell and Carthew, 2000, Martinek and Young, 2000, Lam and Thummel, 2000, Piccin et al., 2001).

To reduce expression of the *FR* gene transcript in all larval tissues, homozygous transgenic flies carrying the FR inverted repeat under the control of UAS (UAS-*FR* RNAi) were crossed with flies that exhibit ubiquitous expression of GAL4 homozygously under the control of the tubulin promoter (tubPGAL4). The change in muscle tonus in response to 1μM DPKQDFMRFamide was significantly attenuated in the larval progeny of this cross (UAS-*FR* RNAi/+; tubP-GAL4/+; Fig 9D). In these progeny, peptide treatment increased muscle tonus by only 9.86 ± 3.45 μN, which was approximately 30% of the response elicited in larvae from both parental control lines (tubP-GAL4/+ genotype and UAS-*FR* RNAi/+ genotype; Fig. 9I; one-way ANOVA, Tamhane's post hoc, $p<0.01$). The response in the progeny (ie. UAS-*FR* RNAi/+ ; tubP-Gal4/+) was only 26% of the Canton-S response (Fig. 9I; one-way ANOVA, Tamhane's post hoc, $p<0.05$). No difference was observed between the DPKQDFMRFamide-induced contractions of the two parental control lines (Fig. 9I; Tamhane's post hoc, $p>0.05$), nor between the parental control lines and Canton-S larvae (Tamhane's post hoc, $p>0.05$), in which expression of the FR gene was not manipulated. Thus, combining the UAS-*FR* RNAi line with GAL4 to ubiquitously disrupt the functionality of FR was successful at inhibiting the peptide's ability to elicit contraction. These results suggest that the FR is at least partially responsible for mediating the myostimulatory effect of peptide on larval body wall muscles.

The *24B*-Gal4 driver (P{GawB}how^{24B}}) expresses Gal4 in all embryonic and larval somatic muscles under the control of regulatory sequences from the *held out wings*

Fig. 9. DPKQDFMRFamide modulates larval muscle tonus via the FMRFamide G-protein coupled receptor (FR). Sample recordings in A and B demonstrate tonic larval body wall muscle contractions in response to application of 1 μM DPKQDFMRFamide (arrows) of two control lines, Canton S and a line containing a single copy of non-activated FR IR construct (UAS-FR RNAi/+), respectively. Trace recordings of peptide-induced muscle contractions from larvae expressing only a single copy of the ubiquitous (tubP-Gal4/+), muscle specific (24B-Gal4/+) or neuron-specific (elav-Gal4/+) Gal4 driver but do not express a UAS-FR IR construct were presented in C, E and G traces, respectively. Traces D, F, and H represent muscle contraction recordings in response to the peptide in larvae in which the UAS-GAL4 system was used to drive the expression of a FMRFamide receptor inverted repeat (FR IR) in ubiquitous (D), muscle tissue specific (F) and pan-neuronal patterns (H). Upward deflections represent phasic contractions. B) Summary of responses to 1 μM DPKQDFMRFamide in each line. The response was attenuated in larvae with the ubiquitous expression of FR IR construct, UAS-*FR* RNAi /+; tubP-GAL4/+ larvae, but not in heterozygous parental control lines, UAS-*FR* RNAi/+ and tubP-GAL4/+ larvae (Tamhane's post hoc, $p<0.01$). Similarly, larvae expressing the FR IR construct specifically in muscle tissue (UAS-*FR* RNAi/+; 24B-GAL4/+) showed a reduced response to the peptide (by ~ 85-90%) when compared to two heterozygous parental control lines, UAS-*FR* RNAi/+ and 24B- GAL4/+ larvae (Tamhane's post hoc, $p<0.01$). Pan-neuronal expression of the FR IR construct in *Drosophila* larvae had no effect on the peptide's ability to modulate muscle tonus. No differences were found between the response of UAS-*FR* RNAi/+; elav-GAL4/+ larvae and their parental controls (UAS-*FR* RNAi/+ and elav-GAL4/+ larvae, One-Way ANOVA, Tamhane's post hoc, $p>0.05$). A triangle indicates significance of $p<0.05$ compared to Canton-S flies. A square represents a significant difference ($p<0.05$) compared to UAS-IR-FR line.


A)
Canton-S
30 µN
1 min
B)
UAS-FR RNAi
C)
tubP-Gal4
D)
UAS-FR RNAi/+; tubP-Gal4/+
E)
24B-Gal4
F)
UAS-FR RNAi/+; 24B-Gal4/+
G)
elav-Gal4
H)
UAS-FR RNAi/+; elav-Gal4/+
I)
Tonus change (µN)
0
10
20
30
40
50
60
Canton-S
(12)
UAS-FR RNAi/+
(10)
tubP-Gal4/+
(10)
UAS-FR RNAi/+; tubP-Gal4/+
(10)
24B-Gal4/+
(10)
UAS-FR RNAi/+; 24B-Gal4/+
(10)
elav-Gal4/+
(13)
UAS-FR RNAi/+; elav-Gal4/+
(10)

gene (*how*) (Schuster et al., 1996). To express the FR-IR construct specifically in muscle cells, and hence disrupt the function of the receptor specifically in muscle tissue, the UAS-*FR*-RNAi flies were crossed with *24B*-GAL4 flies to yield a heterozygous F1 generation (UAS-*FR* RNAi/+; 24B-GAL4/+). To control for potential effects of P-vector insertions present in all larvae, two control lines were used, heterozygous UAS-*FR* RNAi flies with a non-active UAS-*FR* IR construct and heterozygous *24B*-GAL4 lines containing a Gal4 vector only . The change in the body wall muscle tonus of the larvae with RNAi-regulated expression of FR gene (UAS-*FR* RNAi/+; 24B-GAL4/+) in response to the peptide is shown in Fig. 9F. Expression of the FR RNAi in muscle cells reduced the amplitude of the muscle tonus elicited by the peptide to 11% and 15% of the responses in UAS-*FR* RNA and 24B-GAL4 controls, respectively (Fig. 9I, Tamhane's post hoc, $p<0.05$ and $p<0.01$), and to 9% of those recorded from Canton-S larvae (Fig 9I, Tamhane's post hoc, $p<0.05$). There was no difference between the peptide-induced body wall contraction of Canton-S, UAS-*FR* RNA and 24-GAL4 control larvae (Tamhane's post hoc, $p>0.05$) in which expression of the FR gene was assumed to be normal. This suggests that the presence of the FR on the postsynaptic muscle cell is necessary for most if not all of the peptide-induced contraction. This result also corroborates previously reported evidence that the effect of DPKQDFMRFamide on tonus of larval body wall muscles is due to a postsynaptic action (Clark et al., 2008).

To examine the possibility that the tonus change is mediated by presynaptic actions of the peptide, neuron-specific expression of the FR inverted repeat was induced by crossing UAS-*FR* RNAi flies with flies harbouring pan-neuronal GAL4 expression under

the control of a single copy of the *elav*-Gal4 driver. The responses of UAS-FR RNAi /+; elav-GAL4/+ and two control progeny lines, heterozygous UAS-*FR* RNAi and heterozygous elav-GAL4 larvae, to 1μM DPKQDFMRFamide application are shown in Fig 9H, B, G, respectively. DPKQDFMRFamide increased muscle tonus by 35.61 ± 4.18 μN in larvae with pan-neuronal expression of FR IR, which was similar to the change in tonus observed in heterozygous control elav-GAL4 (Fig. 9I; 43.02 ± 8.62 μN, Tamhane's post hoc, $p>0.05$) and UAS-FR-IR larvae (Fig. 9I; 31.96 ± 5.83 μN, Tamhane's post hoc, $p>0.05$) as well as to the response of Canton-S larvae (Fig. 9I; 38.11 ± 4.92 μN, Tamhane's post hoc, $p>0.05$). Pan-neuronal expression of the FR IR construct had no effect on the ability of peptide to increase muscle tonus.

3.2 PLC-IP3 pathway

The results presented above suggest that the effect of DPKQDFMRFamide on body wall muscle tonus is mediated via a GPCR. Binding of a signal molecule to a GPCR results in activation of a G-protein that subsequently modulates the activity of various enzymes, ion channels or transport proteins and usually leads to a change in the production of second messengers. One of the major second messenger molecules that plays an important role in signal transduction is inositol trisphosphate (IP_3). IP_3 is produced by hydrolysis of PIP_2, which is catalyzed by Phospholipase C (PLC). This reaction produces DAG and IP_3, both of which act as second messenger molecules. IP_3, is a small water-soluble molecule whose primary function is release of Ca^{2+} from intracellular stores, primarily endoplasmatic reticulum (ER), in a variety of cells, including muscles (Berridge., 1984, Berridge., 2009, Krauss, 2001). Calcium release from internal

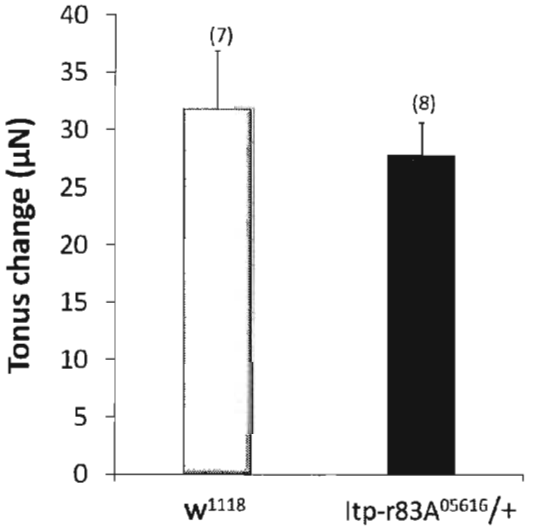
stores is accomplished by the binding of IP_3 to the ligand gated ion channels called IP_3 receptors.

Excitatory effects of RFamides have been previously associated with increasing IP_3 levels in heart tissue of *Lymnea stagnalis* (Willoughby et al., 1999b) and tentacle retractor muscles of *Helix aspersa* (Falconer et al., 1993). In addition, the ability of FMRFamide and FLRFamide to increase tonus in *Lymnea stagnalis* heart was inhibited by approximately 40 % in the presence of 2.5 mM neomycin, a PLC inhibitor (Willoughby et al., 1999b). These observations make the PLC-IP3 pathway an attractive candidate for mediating DPKQDFMRFamide's excitatory effects. If the peptide's effects are indeed mediated via the PLC-IP3 pathway, it would be predicted that the ability of the peptide to increase muscle contraction would be attenuated or abolished by disrupting either PLC or IP_3 receptor (IP_3-R) activity. To investigate this hypothesis, I took advantage of *Drosophila* fly lines in which either PLC or IP_3 receptor functionality had been disturbed with mutations in genes (norpA and Plc21C) that encode for PLCβ or in the single gene known to encode for the IP_3 receptor, *itpr,* respectively.

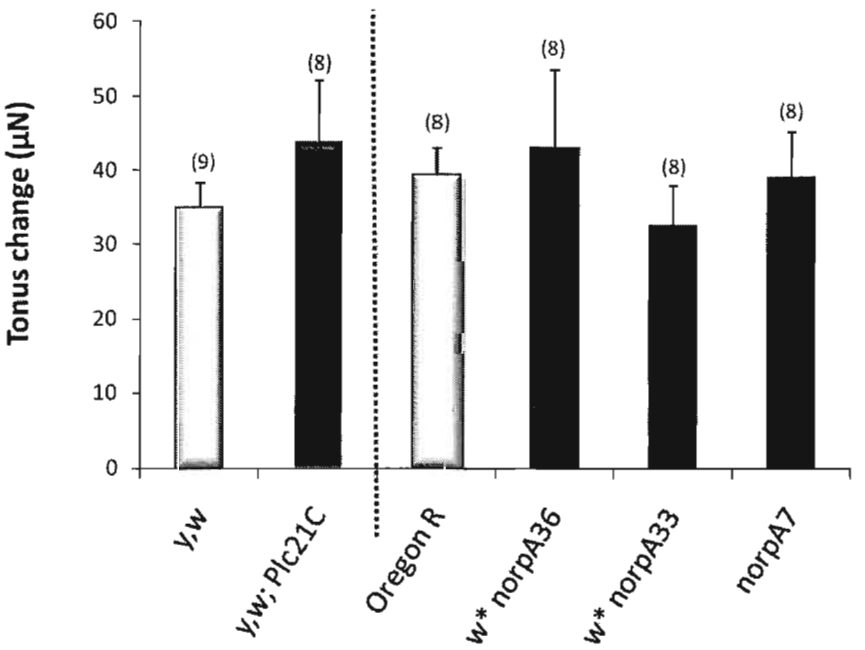
Heterozygous IP_3 receptor mutant larvae (Itp-r83A^{05616}) exhibit approximately 50% reduction in IP3-R transcript levels (Klose et al., 2010). Fig. 10A shows the averaged change in the body wall muscle contraction of heterozygous IP_3 receptor mutants (Itp-r83A^{05616}) and wild type larvae in response to the DPKQDFMRFamide. The mutant larvae responded to peptide with an average muscle tonus increase of 27.74 ± 2.87 μN, which was not significantly reduced from the response elicited by peptide in wild type larvae (t-test, $p > 0.05$).

Fig. 10. Disruption of PLCβ or IP_3 receptor activity had no effect on the ability of DPKQDFMRFamide to modulate muscle tonus in *Drosophila* larvae. Muscle contraction assays were performed by perfusing body wall muscles with 1 μM peptide for 5 minutes and recording the tonus change. The tonus change was calculated as the difference between the maximal rise in tonus within the 5 minute peptide application period and the pre-peptide tonus level. Muscle contraction assays were performed on fly lines carrying mutation on the genes encoding for A) IP_3 receptor (*itpr*) or B) PLCβ (*norpA* or *plc21C* gene). No significant difference was observed between the effect of the peptide on muscle tonus in heterozygous IP_3 receptor mutants (Itp-r83A^{05616}) and wild type larvae (t-test for independent samples, $p>0.05$). The peptide increased muscle tonus in all *norpA* mutants (w* norpA^{33}, w* norpA^{36} and norpA^{7}) and *Plc21C* mutant (y^{1} w^{1118}; Plc21C^{A24}) larvae to a level comparable to their respective controls, Oregon R and y,w. (t-test for independent samples, $p>0.05$).

A)


Tonus change (µN)
40
35
30
25
20
15
10
5
0
(7)
(8)
w1118
Itp-r83A05616/+

B)


Tonus change (µN)
60
50
40
30
20
10
0
(9)
(8)
(8)
(8)
(8)
(8)
y,w
y,w; Plc21C
Oregon R
w* norpA36
w* norpA33
norpA7

Each of the mutants, w* norpA^{33}, w* norpA^{36} and norpA^{7} carries a point mutation in the norpA gene induced by ethyl methanesulfonate mutagenesis on the Oregon-R line. PLC activity is reported to be reduced to approximately 1-1.5 % of wild type levels (Pearn et al., 1996), and PLC activity in norpA^{7} mutants is reduced to about 2–3% of normal levels (Inoue et al., 1988). The *Plc21C* gene mutant, y^{1} w^{1118}; Plc21C^{A24}, had been designed using P element insertion mutagenesis (Bellen et al., 2004). Although it has been reported that the plc21C gene encodes for two transcripts, of which one is expressed in the adult head only and the other is expressed in adult head and body tissue throughout development (Shortridge *et al.,* 1991), the exact levels of their expression in y^{1} w^{1118}; Plc21C^{A24} mutant line are yet to be determined.

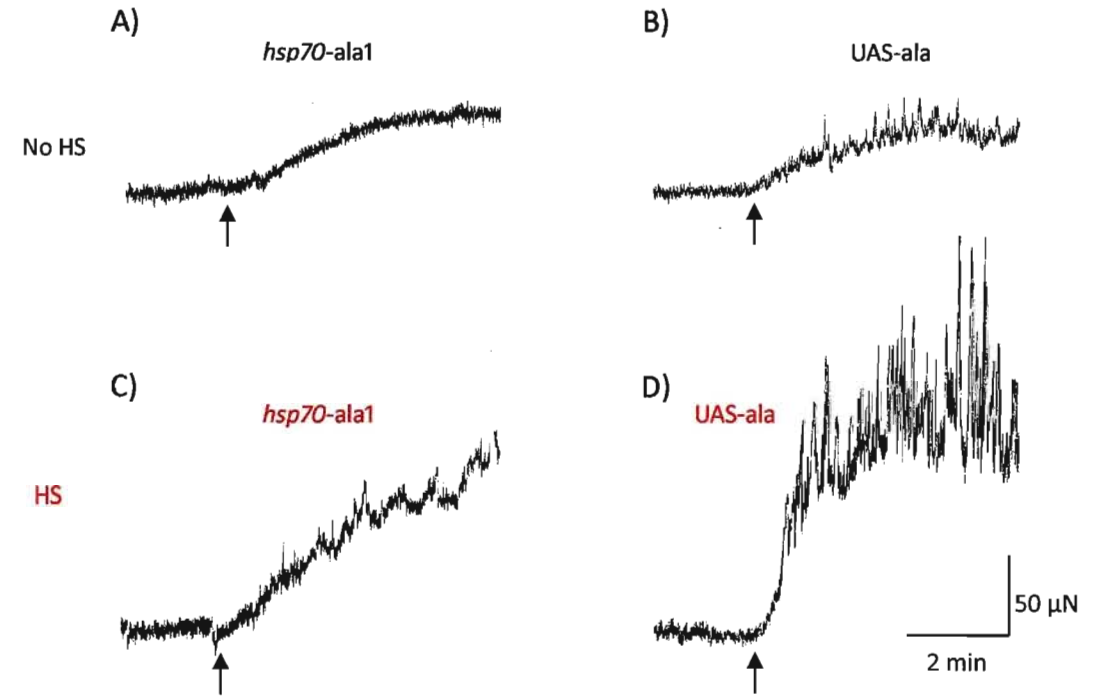
The application of 1 µM DPKQDFMRFamide induced an increase in muscle tonus of larval body wall muscles of all PLC mutants (Fig 10B). The maximal increases in tonus of w* norpA^{36}, w* norpA^{33} and norpA^{7} in response to the peptide were 42.94 ± 10.55 µN, 32.49 ± 5.41 µN and 38.95 ± 6.18 µN, respectively. These changes in muscle tonus were not significantly different from the 39.33 ± 6.03 µN tonus increase observed in wild type, Oregon R larvae (one-way ANOVA, p>o.o5). Similar results were found with plc21C mutant flies. The response to the peptide was not attenuated in the y^{1} w^{1118}; Plc21C^{A24} mutant line when compared to the y,w control line (Fig 10B, t-test, p> 0.05). Together these data indicate that neither PLC nor IP_3 are likely to be involved in mediating myostimulatory effects of DPKQDFMRFamide.

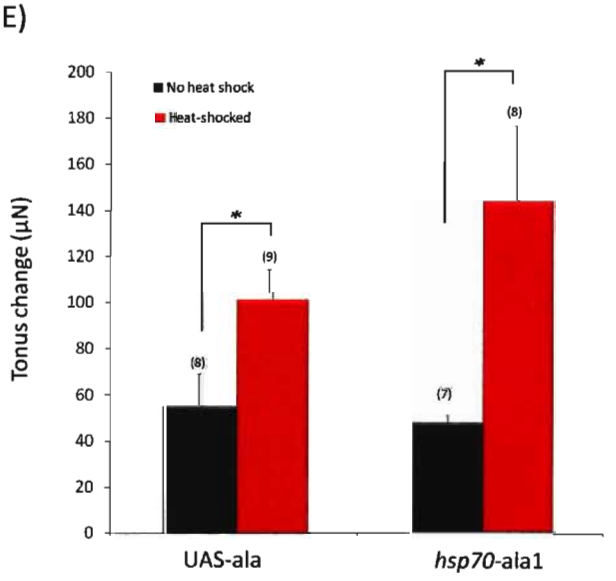
3.3 CaMKII

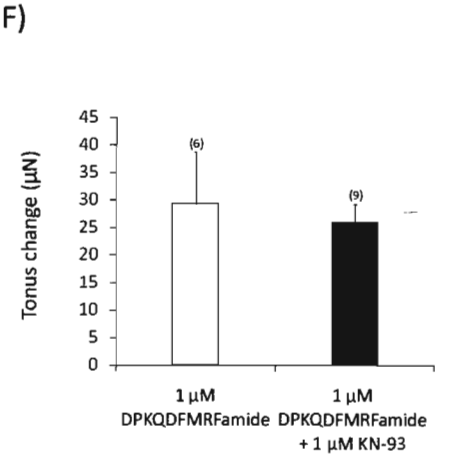
Ca^{2+} ions are important intracellular signals implicated in regulating a number of processes including muscle contraction. The stimulatory effect of DPKQDFMRFamide on body wall muscle contractions requires influx of extracellular Ca^{2+} (Clark et al., 2008), which suggests that an increase in intracellular Ca^{2+} concentration immediately follows the peptide application. Ca^{2+} effects are commonly mediated by Ca^{2+} binding proteins, such as calmodulin (CaM), a small protein (148 amino acids) consisting of two domains able to bind two Ca^{2+} ions each (Krauss, 2001).The effects of CaM are most often mediated by activation of a multifunctional CaM kinase, CaM kinase II, that consists of a dodecameric holoenzyme which gets activated when CaM binds to its autoinhibitory domain. This results in dissociation of the catalytic domain from the autoinhibitory domain and autophosphorylation of a threonine residue downstream from the catalytic subunit (Rosenberg et al., 2005; Hunter & Schulman, 2005).

Both genetic and pharmacological disruption of CaMKII activity was used to test the possibility that CaMKII might mediate the peptide-induced increase in muscle contraction. Genetic disruption of CaMKII activity had been accomplished in the ala1 transgenic line that contains a sequence encoding for the autoinhibitory domain of CaMKII, inserted downstream from a heat shock promoter (Griffith et al., 1993). At room temperature, ala1 flies express a low level of this peptide of 1.9-3.9 uM, which prominently increases following heat shock treatment at 37 °C (Griffith et al., 1993). A control for ala1 flies is the UAS-ala line, which contains the CaMKII inhibitory peptide under the control of a UAS promoter rather than a heat shock promoter. The expression

Fig. 11. Genetic and pharmacological inhibition of CaMKII does not affect the peptide's action on tonus of larval body wall muscles in *Drosophila*. Sample recordings demonstrate the effects of 1 µM DPKQDFMRFamide on spontaneous tonic contractions of body wall muscles in ala1 and UAS-ala larvae . A and B represent the response of ala1 and UAS-ala larvae, respectively, without prior heat-shock treatment, while C and D demonstrate the response of ala1 and UAS-ala larvae, respectively, submitted to a 1 hour heat-shock treatment at 37°C. Peptide was applied at the upward pointing arrows by continuous perfusion. E) Averaged responses from ala1 larvae, and the control, UAS-ala larvae. Both groups showed similar increases in tonus in response to peptide treatment. Exposing larvae to a heat shock treatment (one hour at 37 °C) is known to induce an increase in expression of CaMKII inhibitory protein in ala 1 flies, but not in UAS-ala flies. The response to the peptide was potentiated in both ala1 and UAS-ala larvae after subjecting them to heat shock. No significant difference was observed between the responses in heat-shocked ala1 and UAS-ala larvae (t-test for independent samples, $p>0.05$). F) The averaged change in muscle tonus in Canton-S larvae in the presence of a CaMKII inhibitor. 1 µM KN-93, a CaMKII inhibitor, had no effect on the peptide's ability to increase muscle tonus (t-test for independent samples, $p>0.05$).


A)
hsp70-ala1
No HS
B)
UAS-ala
C)
hsp70-ala1
HS
D)
UAS-ala
50 µN
2 min


E)
No heat shock
Heat-shocked
Tonus change (µN)
200
180
160
140
120
100
80
60
40
20
0
(8)
(9)
(7)
(8)
*
*
UAS-ala
hsp70-ala1


F)
Tonus change (µN)
45
40
35
30
25
20
15
10
5
0
(6)
(9)
1 µM DPKQDFMRFamide
1 µM DPKQDFMRFamide + 1 µM KN-93

of CaMKII inhibitory peptide in UAS-ala flies is not activated until a Gal4 driver is introduced.

Fig 11A and 11B show body wall muscle contractions of ala1 and their respective control (UAS-ala) larvae in response to 1 μM DPKQDFMRFamide application. The non-heat-shocked ala1 larvae showed a similar increase in muscle tonus in response to peptide to that of the control line (Fig 11E; t-test, $p>0.05$). Hence, constitutive expression of the CaMKII inhibitory protein in ala1 larvae had no effect on the DPKQDFMRFamide-induced tonus change. Exposing ala1 flies to an hour-long heat shock treatment at 37°C has been reported to increase CaMKII inhibitory protein expression levels and to decrease CaMKII activity by 70-100% (Griffith et al., 1993). Heat-shock treatment of ala1 larvae completely inhibits stimulatory effects of DPKQDFMRFamide on EJP amplitude (Dunn and Mercier, 2005) of *Drosophila* muscle fibres innervated by type 1b motor neuron boutons. In contrast, the DPKQDFMRFamide-induced muscle contraction is enhanced in heat-shocked ala1 larvae by approximately 150% when compared to non heat-shocked ala1 larvae (Fig 11E, t- test, $p<0.05$). These results suggest that CamKII is not required for the effects of DPKQDFMRFamide on muscle tonus to occur. To test the possibility that the augmented response to the peptide in heat shocked larvae is due to the disruption of CaMKII activity, the peptide was also tested on heat-shocked control larvae. Heat shock also increased the peptide's effect on muscle tonus in these control larvae; the response was increased by approximately 100% compared to the non heat-shocked controls (t-test, $p<0.05$).There was no significant difference between the responses of heat-shocked ala1 and heat-shocked

UAS-ala larvae (t-test, $p>0.05$). These results suggest that the enhanced responses to the peptide are attributable to the heat shock treatment rather than to the CaMKII inhibition.

To further investigate a possible role of CaMKII in modulation of muscle tonus by DPKQDFMRFamide, a pharmacological inhibitor of CaMKII activity was used (Fig. 11F). KN-93, a cell permeable CaMKII inhibitor, was applied to the preparation at a concentration of 1μM for 20 minutes, and immediately thereafter the preparation was exposed to a solution containing 1μM DPKQDFMRFamide and 1μM KN-93. The concentration of KN-93 used in the experiment is above the IC_{50} level of 0.37μM as reported by Sumi et al. (1991) and was shown to inhibit the peptide's ability to increase EJP amplitude (Dunn and Mercier, 2005). There was no significant difference between the muscle tonus change elicited by 1μM DPKQDFMRFamide alone (29.13 ± 9.59 μN) or in presence of KN-93 (25.84 ± 3.27 μN) (t-test, $p>0.05$).

3.4 Cyclic AMP and cyclic GMP pathways

3.4.1 Effect of DPKQDFMRFamide on cyclic AMP and cyclic GMP levels.

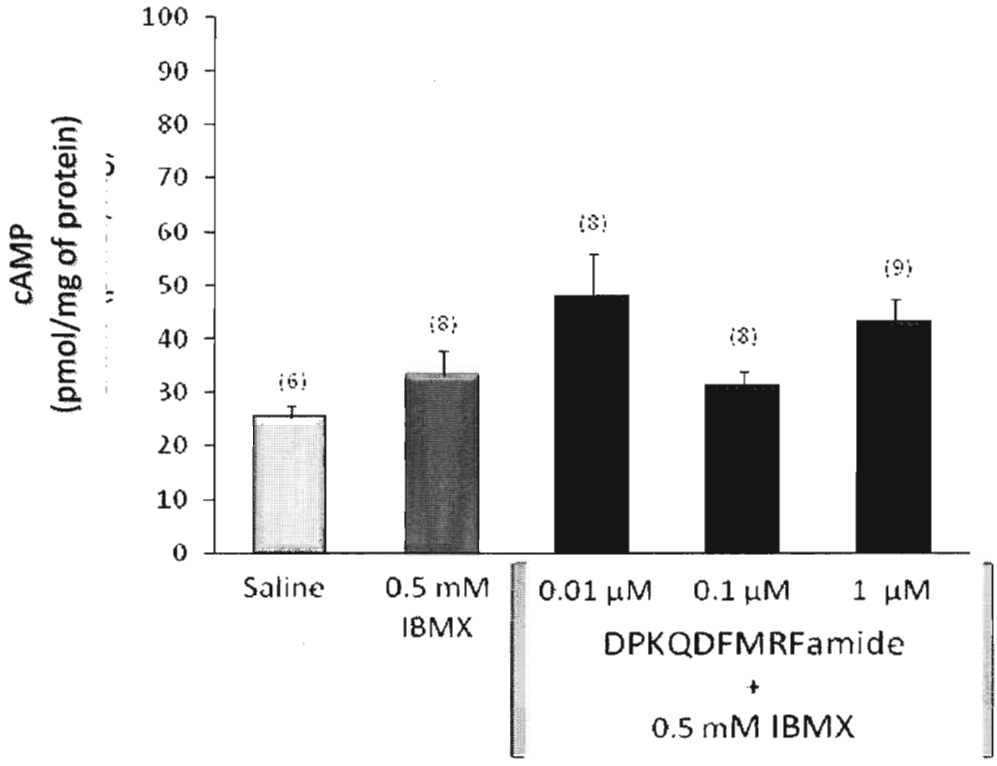
The modulatory effects of neuropeptides on muscle contractions have been associated with the ability of cAMP to change the excitability of the cell by modulating membrane-bound ion channels (Erxleben et al., 1995; Bishop et al., 1991,1987). Proctolin, for example, is able to enhance muscle contractions by increasing membrane resistance through cAMP-mediated closure of non-voltage dependent K^+ channels (Erxleben et al., 1995) and by modulating the activity of Ca^{2+} channels via a cAMP – dependent pathway (Bishop et al., 1991,1987). Since the ability of DPKQDFMRFamide to

induce body wall muscle contractions requires activation of L-type Ca^{2+} channels, it is plausible that this peptide activates the cAMP pathway which, in turn, results in the activation of VGCC. To investigate whether or not cyclic nucleotides are involved in mediating the action of the peptide on larval body wall muscles, cAMP and cGMP levels in body wall muscles were determined with enzyme immunoassay after incubating the tissues in various peptide concentrations in the presence of isobutylmethylxanthine (IBMX), a phoshodiesterase inhibitor, known to slow the breakdown of both cAMP and cGMP (Beavo et al., 1970, Beavo & Reifsnyder 1990; Goy, 1990).

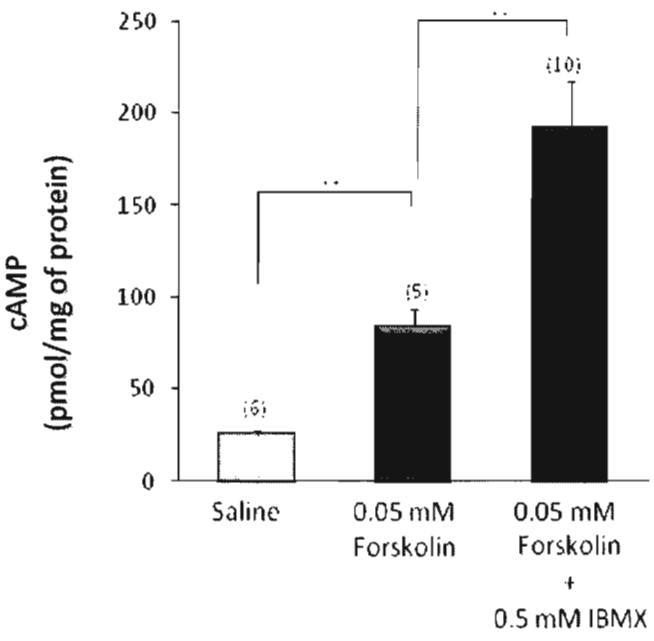
Following the exposure of body wall muscles to DPKQDFMRFamide at concentrations ranging from 10 nM to 1μM in the presence of 0.5 mM IBMX, cAMP did not differ from the control level (33.0 ± 4 pmol of cAMP/mg protein) measured in IBMX alone (Fig 12A, One-Way ANOVA, Tamhane's post hoc, $p>0.05$). There was also no significant difference between cAMP levels following the exposure of larval muscles to 0.5 mM IBMX alone and saline alone (Fig 12A, one-way ANOVA, Tamhane's post hoc, $p>0.05$), which raised a concern that this concentration of a non-specific phoshodiesterase inhibitor, IBMX, is not sufficient to increase cAMP levels in *Drosophila* larval muscles. To confirm that the IBMX concentration was sufficient to slow the breakdown of cAMP, forskolin, an adenylate cyclase activator, was added in the presence and absence of 0.5 mM IBMX. When compared to saline levels, treatment with 0.05 mM forskolin alone increased cAMP levels by approximately 230% (Fig 12B, one-way ANOVA, Tamhane's post hoc, $p<0.01$). In the presence of 0.5 mM IBMX,

Fig. 12. DPKQDFMRFamide does not affect cAMP levels in *Drosophila* body wall muscles. A) The effects of DPKQDFMRFamide on cAMP levels in larval body wall muscles in the presence of 0.5 mM IBMX. cAMP levels following a 10 min incubation of *Drosophila* body wall muscles in 10 nM, 100 nM and 1μM DPKQDFMRFamide in the presence of 0.5 mM IBMX did not exceed the cAMP level observed after incubation in 0.5 mM IBMX alone. B) 0.05 mM forskolin in the presence of 0.5 mM IBMX increased cAMP levels in larval body wall muscles by approximately 130% of the level observed in forskolin alone condition. Thus, IBMX potentiated the effects of forskolin on cAMP levels.

A)


cAMP
(pmol/mg of protein)
100
90
80
70
60
50
40
30
20
10
0
(6)
(8)
(8)
(8)
(9)
Saline
0.5 mM IBMX
0.01 µM
0.1 µM
1 µM
DPKQDFMRFamide
+
0.5 mM IBMX

B)


cAMP
(pmol/mg of protein)
250
200
150
100
50
0
(6)
(5)
(10)
Saline
0.05 mM Forskolin
0.05 mM Forskolin
+
0.5 mM IBMX

forskolin increased cAMP levels by approximately 650 % (to 191.6 ± 25.8 pmol/mg of protein), which is approximately a 2.3 fold increase from the cAMP levels obtained with forskolin alone (83.9 ± 9.5 pmol/mg of protein) (Fig 12B, One-Way ANOVA, Tamhane's post hoc, $p < 0.01$). The data suggest that 0.5 mM IBMX is sufficient to act synergistically with other cAMP elevating compounds to increase cAMP levels.

The effect of the peptide on cGMP levels was also investigated. One-way ANOVA revealed a significant difference in cGMP levels between treatment groups ($p<0.01$). Fig 13 shows that treatment with IBMX alone resulted in a 260 % increase in cGMP level when compared to saline treatment (Tamhane's post hoc, $p<0.01$), bringing the level of cGMP to 3.66 ± 0.31 pmol/mg. Following treatment with 10 nM, 100 nM and 1μM DPKQDFMRFamide in the presence of 0.5 mM IBMX, the cGMP levels were 4.04 ± 0.74, 3.75 ± 0.47 and 3.60 ± 0.33 pmol/mg, respectively (Fig 13). These levels were not significantly different from the cGMP level obtained with IBMX alone (Tamhane's post hoc, $p>0.05$). These data suggest that cGMP does not play a role in mediating the peptide's effects on muscle tonus.

3.4.2 Effects of IBMX, forskolin and protein kinase inhibitors on peptide's activity

To further investigate the possible role of cyclic nucleotide signal transduction pathways, the effects of pharmacological agents known to modulate the activity of different components of these pathways on physiological actions of the peptide were tested.

IBMX, a broad-spectrum cAMP and cGMP phosphodiesterase inhibitor (Beavo, et al., 1970), was used to determine whether slowing the breakdown of cyclic nucleotides

would potentiate the effect of a low peptide dose on contraction. Forskolin, an adenylyl cyclase activator (IC_{50} =4 μM) was used to determine whether or not elevating cAMP level would mimic the physiological response to the peptide. Protein kinase inhibitors were used to determine whether blocking cAMP- and cGMP-dependent protein kinases inhibits peptide-induced contractions.

Previously published data showed that DPKQDFMRFamide increases muscle tonus of *Drosophila* larvae in a dose-dependent manner, with a threshold concentration of approximately 0.1 μM (Clark et al, 2008). When DPKQDFMRFamide was applied at this threshold level (0.1 μM), the body wall muscles responded with a very small increase, 11.6 ± 4.3 μN, in basal tonus. This response was not significantly increased in the presence of 0.5 mM IBMX (Fig 14A, t-test $p>0.05$). Treatment with IBMX alone (n=7), or forskolin alone resulted in only minimal changes in body wall muscle tonus of 1.1 ± 7.3 μN and 11.6 ± 4.7 μN, respectively. The primary cellular targets of intracellular cAMP and cGMP are the cAMP-dependent protein kinase (PKA) and the cGMP-dependent protein kinase (PKG), respectively. Hence, I also tested whether or not a PKA inhibitor, Rp-cAMPs (IC_{50} =4.9 ± 2.1 μM, Schaap, 1993; K_i =11μM, Rothermel and Botelho, 1988) and a potent cell permeable PKG inhibitor, Rp-8-pCPT-cGMPS (K_i =0.5μM, Butt et al., 1994) would antagonize the peptide's effect on muscle tonus. Neither Rp-cAMPS (100 μM) nor Rp-8-pCPT-cGMPS (10μM) significantly attenuated the response to 1 μM DPKQDFMRFamide (Fig 14B, t-test, $p>0.05$). When applied alone, the inhibitors did not alter muscle tonus.

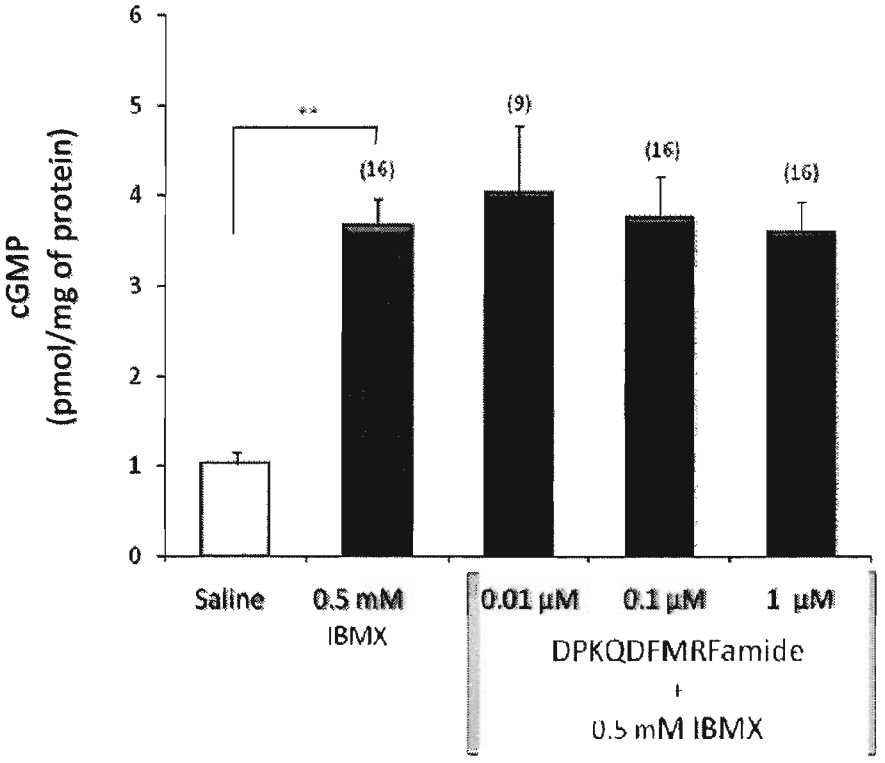
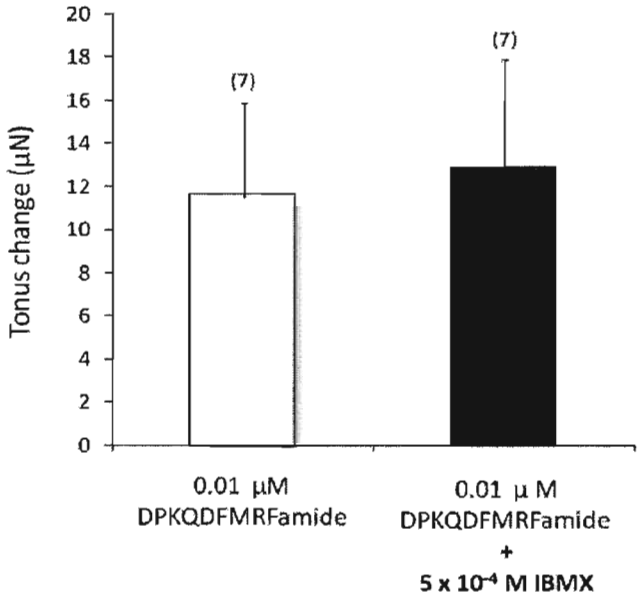


Fig. 13. DPKQDFMRFamide does not affect cGMP levels in *Drosophila* body wall muscles. The effects of DPKQDFMRFamide on cGMP levels in larval body wall muscles in the presence of 0.5 mM IBMX. cGMP levels following 10 min incubation of *Drosophila* body wall muscles in 10 nM, 100 nM and 1µM DPKQDFMRFamide in the presence of 0.5 mM IBMX did not exceed the cGMP level observed after incubation in 0.5 mM IBMX alone.

A)



B)

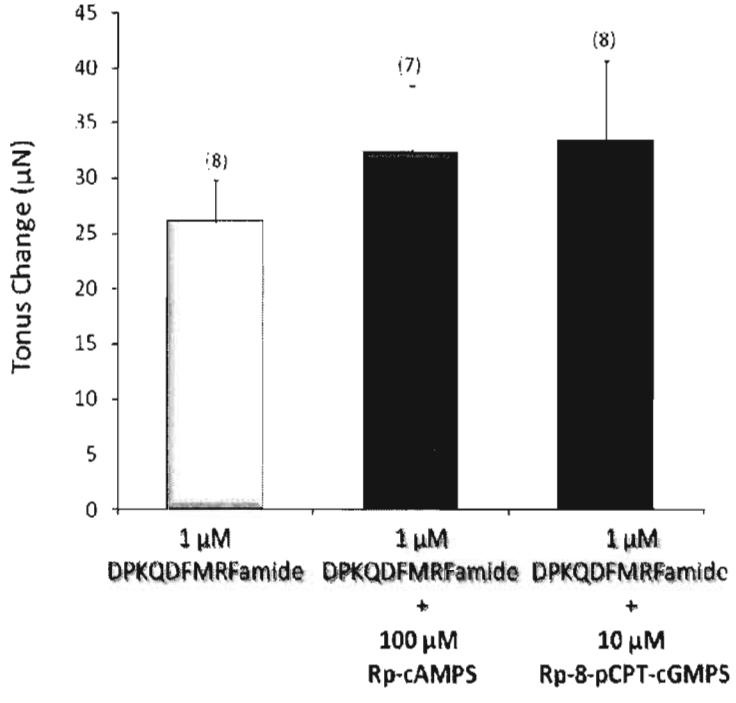


Fig. 14. The application of A) 0.5 mM IBMX, a broad-spectrum phosphodiesterase inhibitor and B) 100 μM Rp-cAMPS and 10 μM Rp-8-pCPT-cGMPS, selective cAMP and cGMP dependent protein kinase inhibitors, had no effect on the actions of peptide on the tonus of *Drosophila* body wall muscles.

4. DISCUSSION

4.1 DPKQDFMRFamide acts via FR GPCR

Previous work showed that DPKQDFMRFamide induces larval body wall muscle contractions and increases muscle tonus in a dose-dependent manner (Clark et al., 2008), but did not identify the cellular targets mediating this effect. To date two major classes of FMRFamide receptors have been identified, a FMRFamide-activated Na^+ channel (FaNaCh) that can be blocked by amiloride (Linguella et al., 1995), and a FMRFamide G-protein coupled receptor (Cazzamali and Grimmelikhuijzen, 2002, Meeusen et al., 2002). Our results suggest that the effect of the *Drosophila* peptide, DPKQDFMRFamide, on the larval body wall muscle contractions are mediated via the dFMRFamide G protein coupled receptor. The UAS/Gal4 system (Brand and Perrimon, 1993) was used to drive the expression of a FMRFamide receptor inverted repeat (*FR* IR) in a tissue-specific manner to silence the receptor gene. When the expression of the FR gene (CG2114) was silenced specifically in muscle cells, the excitatory effect of DPKQDFMRFamide on muscle tonus was reduced by 85-90 % (Fig 1B), and when the gene expression was silenced in all tissues, the response was reduced by approximately 70% (Fig 1A). This 15-20 % difference observed between the reduction in response of larval muscles with ubiquitous and muscle-specific receptor knock-down might reflect different levels of silencing achieved in these fly lines. However, the excitatory effect of the peptide on larval muscles was not completely abolished in either case. If receptor expression was completely silenced, and if the peptide acts through the FMRFamide-GPCR only, then silencing of the FR gene ubiquitously would completely abolish the

ability of peptide to increase muscle tonus. However the *FR* RNAi larvae with the Gal4 driven in all tissues still responded to 1μM DPKQDFMRFamide with an average increase in muscle tonus of 9.8 μN (Fig 9D). At least two possibilities can account for this result. First, the larvae used for physiological recordings were heterozygous for the Gal4 and UAS-RNAi element. Thus, it is likely that expression of the receptor gene was not knocked down completely, and that larval cells retained sufficient expression of the dFMRFamide receptor to mediate a weak response to the peptide. An alternative possibility is that DPKQDFMRFamide may act via targets other than the FMRFamide receptor. Using the β-*arrestin2*-green fluorescent protein (βarr2-GFP) translocation assay, Johnson et al. (2003) showed that 100 nM DPKQDFMRFamide activates myossuppresin receptors expressed in Human Embryonic Kidney (HEK) cells which had been simultaneously transfected with a G protein receptor kinase (*GRK2*) to enhance the receptor phosphorylation and arrestin enrolment. Hence, it may be possible that DPKQDFMRFamide is acting through more than one receptor to elicit muscle contraction.

4.2 Second messenger pathways

4.2.1 cAMP and cGMP pathways. The ability of DPKQDFMRFamide to increase *Drosophila* larval muscle tonus is completely abolished by nifedipine and nicardipine (Clark et al., 2008), suggesting that L-type calcium channels are necessary for the response to occur. One mechanism underlying the peptide's myotropic effect, therefore, may involve peptide upregulation of Ca^{2+} influx through voltage-gated Ca^{2+} channels. This mechanism is not novel and is utilized, for example, by the myotropic

neuropeptide proctolin in the crayfish tonic flexor muscle (Bishop et al. 1991) and by the small cardioactive peptides (SCPs) and myomodulins (MMs) in the accessory radula closer muscle of the marine snail *Aplysia californica* (Brezina et al. 1994). cAMP has been shown to modulate L-type calcium currents in mammalian (Catterall,2000) , crustacean (Bishop et al., 1991,1987), and insect (Bhattacharya et al., 1999) muscles, presumably via PKA-dependent phosphorylation of L-type Ca^{2+} channels. On the other hand, cAMP was also found to mediate the modulation of muscle contractions by a number of neuropeptides (Erxleben et al., 1995; Bishop et al., 1991,1987, Trim et al., 1998) including FMRFamide (Willoughby et al., 1999a), and biogenic amines such as dopamine (Knotz & Mercier, 1995) and octopamine (Clark & Lange, 2003).

cGMP has been shown to modulate calcium channel activity (Taghuchi et al., 1997) as well, although by inhibiting Ca^{2+} channels. Although cGMP has not been implicated in the modulatory functions of FMRFamide-like peptides on muscle contraction, it has been found to be involved in mediating their effects on synaptic transmission (Badhwar et al., 2006). This suggested that pathways involving cyclic nucleotides might play a role in mediating the effects of the peptide on *Drosophila* larval muscle contractions including FMRFamide.

Several independent lines of evidence presented in this study suggest that cAMP, cGMP and their respective protein kinases, PKA and PKG, are not required for the peptide to increase muscle tonus in *Drosophila* larvae. First, the peptide, in the presence of a phoshodiesterase inhibitor, IBMX, did not increase cAMP or cGMP levels in *Drosophila* larval muscles. Second, IBMX failed to potentiate the peptide's effect on

contraction. Third, inhibitors of cAMP-dependent and cGMP-dependent protein kinases failed to reduce peptide-induced contractions. Fourth, neither IBMX nor forskolin mimicked the peptide's effect. These observations suggest that changes in cAMP or cGMP are neither necessary nor sufficient to mediate the peptide's effect on muscle tonus.

Basal cAMP levels in larval muscle tissue (in the absence of IBMX) were approximately 25 pmol/mg of protein. At 50 μM forskolin increased cAMP levels by 130% from basal levels, and this response was potentiated by the presence of 0.5 mM IBMX, a non-selective cAMP/cGMP phosphodiesterase inhibitor. Similar results have been reported for locust skeletal and visceral muscles (Lange and Orchard, 1986). Since cAMP levels in the presence of both forskolin and IBMX were greater than the levels obtained with either forskolin alone or IBMX alone (Fig 4a,b), the IBMX concentration appears to be sufficient to reduce cAMP breakdown and generate a synergistic effect with forskolin. In contrast to the effects of forskolin, DPKQDFMRFamide in the presence of IBMX did not increase cAMP levels above those seen with IBMX alone, suggesting that DPKQDFMRFamide does not act by stimulating cAMP production. Also, DPKQDFMRFamide in the presence of IBMX did not increase cGMP levels above those seen with IBMX alone. This suggests that changes in cAMP and cGMP levels are not necessary for the peptide's action on muscle contractions.

An interesting finding was the difference between the basal cAMP levels in *Drosophila* larval muscle tissue (25 pmol/mg of protein) reported in this study and the basal cAMP levels reported in the *Drosophila* adult tissues and tissues of other insects.

The cAMP levels measured in this study appear to be 13-16 fold higher than cAMP levels found in the whole adult female CS flies (1.5-1.6 pmol/mg of adult fly, Byers et al., 1981) and in the brain of 3-day-old flies (1.87 ± 0.04, Xu et al., 1998) using a radioimmunoassay technique. Levels of cAMP in the present study are also drastically higher than in *Diploptera punctata* Malpighian tubules (2.39±0.26, pmol cAMP/mg protein, Tobe et al., 2005), locust visceral muscle (4.19 ±0.33 pmol cAMP/mg protein, Lange and Orchard., 1986) and locust skeletal muscle (1.85 ± 0.3 pmol cAMP/mg protein, Evans, 1984). On the other hand, the basal levels of cGMP (~1 pmol/mg of protein) in larval body wall muscles were similar to basal cGMP levels (0.5 , 0.45 and 1.9 pmol/mg, respectively) reported in locust skeletal muscles (Evans, 1984), abdomen of the adult giant silkmoth *A.polyphemus* (Schwartz and Truman, 1984) and lobster skeletal muscles (Goy et al., 1987). The cause of the discrepancy between previously reported basal cAMP levels in insect muscles and the levels found here is not clear. However, larval body wall muscles used for measuring basal cAMP and cGMP levels in this study were incubated in HL6 saline, which is rich in amino acids. One could then speculate that the presence of amino acids in HL6 saline can act to increase cAMP levels. The fact that basal cGMP levels are similar to those reported previously suggests that amino acids do not work to increase guanylyl cyclase (GC) activity. An alternative possibility is that the cAMP turnover rate in larval muscles is different from those observed in the muscle tissue of adult insects, since higher levels of basal cAMP levels (72 pmol/mg of protein) have been previously reported in *Drosophila* larval muscle fibres (Cheung et al., 1999).

To provide additional evidence, I also tested the effects of pharmacological agents that activate or inhibit components of cAMP and cGMP pathway on the ability of the peptide to increase muscle tonus. The peptide-induced contracture was not potentiated by IBMX, a non-selective cAMP/cGMP phosphodiesterase inhibitor, nor was the response to the peptide mimicked by IBMX or forskolin. Since the concentration of forskolin increased cAMP levels in the tissue, changes in cAMP do not appear to be sufficient to elicit contracture.

The PKA inhibitor, Rp-cAMPS and the PKG inhibitor, Rp-8-pCPT-cGMPS, were also used to examine the possible role of cAMP and cGMP pathways in the peptide-induced contractions. The concentrations of Rp-cAMPs (100 μM) and Rp-8-pCPT-cGMPS (10 μM) used in this study were shown to successfully reduce the ability of DF_2, a crayfish FMRFamide-like peptide, to enchance EJP amplitude by 76 % and 40 % ,respectively (Badhwar et al., 2006). In the present work, neither kinase inhibitor altered the ability of DPKQDFMRFamide to induce an increase in muscle tonus.

4.2.2 PLC-IP3 pathway. IP_3 has been implicated in mediating the excitatory effects of several neuropeptides, including FMRFamide (Willoughby et al., 1999b, Falconer et al., 1993) and proctolin (Hinton & Osborne, 1995, Baines et al.,1990), on muscle contractions.

Since DPKQDFMRFamide increases muscle tonus with a relatively slow time rise course and induces irregular phasic muscle contractions (Clark et al., 2008), the regulation of intracellular Ca^{2+} may be of importance for the peptide's effects. One of the main signalling molecules regulating Ca^{2+} oscillations in the cell is IP_3 which

stimulates the release of Ca^{2+} from the intracellular stores via IP_3 receptors (Berridge, 1984, 2009).

To test this hypothesis, muscle tonus was recorded in several mutants with disturbances in functionality of molecular components of the PLC-IP_3 pathway. Although several PLC types are present in the cell, only PLC-β is activated by a G protein coupled receptor (Vauquelin & von Mentzer, 2007; Krauss, 2001). In *Drosophila melanogaster* two genes encode for PLC-β; norpA and plc-21c. norpA mutants exhibit 97-99 % reduction in PLC activity compared to control flies, as determined by *in vitro* PLC assays of eye and whole head homogenates (Inoue et al., 1988, Pearn et al., 1996). Unfortunately, the level of PLC activity in tissues of Plc-21C mutants remains unknown. I tested the effects of 1 μM DPKQDFMRfamide on muscle tonus of several norpA mutants, w* $norpA^{33}$, w*$norpA^{36}$ and $norpA^{7}$, and one PLC-21C mutant. Peptide-induced contractions in these mutants were neither reduced nor augmented when compared to control larvae suggesting that PLC-IP_3 pathway is not involved in mediating the peptide's effect on muscle contacture.

One could argue that the estimated PLC activities from the adult head tissues of norpA mutants do not necessarily represent the activity of the PLC in the rest of the norpA adult tissues or larval tissue, especially since PLC-β is encoded by two different genes. This, together with the fact that the PLC activity of PLC-21C mutants is unknown, brings into question the interpretation of the present data. However, two lines of evidence suggest that PLC activity in norpA mutants is not confined to the CNS. The first stems from the molecular and genetic studies of PLC-β encoding genes. Although early

molecular characterization and physiological studies indicated that norpA expression is confined to the retina (Bloomquist et al., 1988), later immunodetection of norpA protein via Western blots showed the presence of a 130 kDa protein in the leg, thorax and abdomen of adult flies (Zhu et al.,1993), suggesting that PLC-β also plays an important role in transduction pathways outside the CNS. Similarly, hybridization of *plc-21* cDNA probes to RNA blots revealed that this gene encodes two transcripts, 6.6 kb and 7 kb long, the smaller of which was detected throughout larval development and in adult heads and bodies (Shortridge et al., 1991). The second line of evidence that norpA gene function is disturbed in tissues other than eye and CNS comes from the physiological studies on norpA mutants. To date, *Drosophila* norpA (*no receptor potential*) mutants have not only been successfully used to elucidate the function of PLC in phototransduction (Pak and Shortridge, 1991), but also in modulation of epithelial fluid transport (Pollock et al.,2003) and larval muscle L-type Ca^{2+} channels currents (Gu and Singh., 1998). For example, fluid transport assays performed on Malpighian tubules showed approximately 60% reduction in neuropeptide-stimulated secretion rate in $norpA^{36}$ mutants compared to wild type flies (Pollock et al.,2003), suggesting that norpA function is not confined to the phototransduction pathway of adult flies. The L-type Ca^{2+} current is reduced in muscle tissue of $norpA^{36}$ larvae by 20 %, hence comprising 80% of the wild type Ca^{2+} current (Gu and Singh., 1997), suggesting that norpA also plays an important role in intracellular signalling in larval muscle tissue.

When PLC is activated, it stimulates the production of IP_3, which then binds to IP_3 receptors present on internal Ca^{2+} stores and releases Ca^{2+} into the cytosol. The results

obtained with norpA mutants, suggest that IP_3 is not involved in mediating peptide-induced contractions. This result is corroborated by the ability of peptide to also induce contracture in IP_3 receptor mutants. Only one gene in *Drosophila*, *itpr-83,* encodes for an IP_3 receptor (IP3R) (Hasan and Rosbash, 1992).

A reverse-transcription-based competitive polymerase chain reaction assay (RT-QPCR) detected itpr-83 gene expression in heads, eyes, antennae and legs of adult flies, from which the expression was most strongly pronounced in antennae, suggesting that the IP_3 receptor may have a role in olfaction (Hasan and Roosbash, 1992). Confocal microscopy, utilized to trace the expression of fluorescent compounds specific for RyR (TX-R-BODIPY-ryanodine), IP3R (FL-Heparin), and thapsigargin-sensitive SERCA (FL-BODIPY-thapsigargin), demonstrated abundant localization of IP_3 receptor protein in the cells of epidermal, muscle and digestive tract tissue in late embryonic stage and adult flies (Vázquez-Martínez ., 2003). The expression studies suggest that IP_3 plays an important role in variety of tissues. Larvae of this particular mutant were shown to express 50% reduction in itpr-83 mRNA levels when compared to wild type larve (Klose et al. 2010). Although the stimulatory effects of DPKQDFMRFamide on peak Ca^{2+} levels in presynaptic terminals (Klose et al., 2010) and on EJP amplitude (Klose et al., 2010; Dunn, 2003) are abolished in IP_3 receptor mutants, the ability of the peptide to increase muscle tonus was not affected in the present work. This suggests that IP_3 is not necessary for the peptide to induce contracture postsynaptically.

4.2.3 CaMKII. Previously published evidence suggests that influx of Ca^{2+} through L-type calcium channels is necessary for DPKQDFMRFamide to increase muscle

contractions (Clark et al.,2008). This interpretation is based on the observation that peptide-induced contractions are drastically reduced in Ca^{2+} free saline or by L-type channel blockers. Currently it is not known if DPKQDFMRFamide causes membrane depolarization that could increase the opening of the voltage-gated Ca^{2+} channels. Some influx of Ca^{2+} presumably leads to an increase in intracellular Ca^{2+} levels; however, it is not clear which effectors act downstream of Ca^{2+} influx to enhance contracture.

CaMKII appears as a likely effector that could mediate the effect of the peptide on muscle contracture from at least two standpoints. First, a previous study reported CaMKII necessary for DPKQDFMRFamide, the same peptide tested in this study, to increase the amplitude of EJPs, and hence necessary for the neuropeptide's stimulatory effects on synaptic transmission to occur (Dunn and Mercier, 2005). Secondly, CaMKII has been implicated in regulating several muscle proteins involved in regulation of calcium levels (Jahn et al., 1988, Dzhura et al., 2000) and muscle contractility (Phietzer, 2001, Sutherland and Walsh, 1989). For example, treatment with CaMKII inhibitors abolishes Ca^{2+} induced enhancement of L-type calcium currents in isolated toad smooth muscle cells (McCarron et al., 1992) and rabbit ventricular myocytes (Anderson et al., 1994) most likely by the direct phosphorylation of the 55-kDa and the 165-kDa protein of voltage gated (L-type) calcium channels (Jahn et al., 1988; Dzhura, 2003). In addition, CaMKII is able to regulate the release and reuptake of Ca^{2+} from internal stores by modulating the activity of RYR channels and by increasing the activity of SERCA, a Ca^{2+} ATPase pump that is involved in sequestering Ca^{2+} from the cytoplasm (for review see Grueter et al., 2007) . In the smooth muscles, the regulatory light chain of myosin is

phosphorylated by Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK), which leads to the activation of myosin ATPase and muscle contraction. Inhibition of CaMKII by KN62 abolished the phosphorylation of MLCK in isolated bovine tracheal smooth muscle cells, which was associated with a decrease in the Ca^{2+} sensitivity of regulatory MLCK phosphorylation. This mechanism was proposed to lead to desensitization of smooth muscle to subsequent contractile stimuli (Pfitzer, 2001). Contrarily, phosphorylation of smooth muscle protein caldesmon by CAMKII prevents its binding to myosin, which may lead to an increase in cross bridge cycling and generation of contractile force (Sutherland & Walsh, 1989).

This study reports that ala1 larvae, exhibiting 30-40% reduction in CaMKII activity (Griffith et al, 1993), respond to DPKQDFMRFmamide with a tonus change comparable to the control, UAS-ala larvae, exhibiting normal levels of CamKII activity. Even after ala1 larvae had been submitted to a heat-shock to reduce the CaMKII activity even further (by approximately 70-100%), the response to the peptide was similar to the heat shocked UAS-ala1 larvae. This suggests that modulation of muscle contractions by the peptide does not require CAMKII.

Contrary to what would be predicted, the response to DPKQDFMRFamide was actually potentiated in heat-shocked ala1 and UAS-ala larvae by approximately 150 and 100 %, respectively. Drummond et al. (1986) reported that heating single fura-2 loaded *Drosophila* larval salivary gland cells to 35°C resulted in a 10-fold increase in intracellular Ca^{2+} concentration even in the Ca^{2+}-free saline, suggesting that a large portion of the increase comes from releasing Ca^{2+} from internal stores. The recovery of $[Ca2+]_i$ was

relatively slow, starting approximately 45 min after the bath temperature had cooled to 25°C (Drummond et al., 1986). Assuming that heat shock affects Ca^{2+} concentration in the muscle cells in a similar way, an increase in intracellular Ca^{2+} after the heat shock treatment could account for the augmentation of the response to the peptide observed following the heat shock treatment.

Although the ala1 transgenic fly line has been used successfully to deduce CaMKII functions in previous electrophysiological (Griffith et al., 1994, Dunn and Mercier, 2005) and behavioural assays (Griffith et al., 1993, Joiner and Griffith, 1997, Broughton et al., 2003), some limitations may apply when it is used in muscle contraction assays. At room temperature, ala1 flies have very low expression of CaMKII inhibitory peptide, and hence it is possible that the residual CaMKII concentration is sufficient to respond to peptide-dependent Ca^{2+} influx and modulate its effectors (Griffith et al., 1993). Heat shock is reported to significantly increase levels of CaMKII inhibitory peptide (Griffith et al., 1993), but at the same time it also increases the intracellular Ca^{2+} levels (Drummond et al., 1986). Thus, a subtle dependence on CaMKII may be masked by the effects that overabundance of intracellular Ca^{2+} exerts on muscle contractions.

To confirm the findings from ala1 flies, I also tested the effects of a pharmacological CaMKII inhibitor, KN-93, on the DPKQDFMRFamide-induced muscle contractions. The application of KN-93, at the same concentration and incubation time at which it abolished the peptide's ability to enhance EJP amplitude (Dunn and Mercier, 2005), did not block the peptide's ability to increase muscle contractions.

4.3 Presynaptic vs postsynaptic effects

Two lines of evidence from this study support the idea that the increase in tonus of *Drosophila* larval body wall muscles is mediated by direct actions of DPKQDFMRFamide on the muscle fibres and not by increasing spontaneous release of neurotransmitters from nerve terminals.

The first comes from experiments in which flies were manipulated genetically in a manner that should reduce dFMRFamide receptor gene expression. The response to 1 μM DPKQDFMRFamide was severely attenuated in larvae designed to reduce dFMRFamide receptor expression specifically in muscle tissue (24B-Gal4/+; UAS-FR RNAi/+), but not in larvae designed to reduce dFMRFamide receptor expression specifically in neurons (elav-Gal4/+; UAS-FR RNAi/+). This suggests that the expression of the dFMRFamide receptor on the postsynaptic cell is necessary for this response to the peptide to occur, and that the expression of this same receptor on presynaptic nerve terminals is not sufficient to allow the peptide to increase muscle tonus.

The second line of evidence comes from experiments investigating second messenger systems that mediate the peptide's actions. The increase in muscle tonus does not appear to require CaMKII activity or the IP_3 receptor. Previous work, however, has shown that DPKQDFMRFamide acts presynaptically via the IP_3 receptors and CaMKII to increase the amplitude of EJPs (Dunn, 2004; Dunn and Mercier, 2005). This effect on EJP amplitude is completely abolished in heat shocked ala1 larve which express CaMKII inhibitory peptide and by KN-93, a pharmacological CAMKII inhibitor (Dunn & Mercier,2005). In addition, the peptide's ability to increase EJP amplitude was

completely abolished in two IP_3 receptor mutants (Dunn, 2004; Klose et al.,2010). Taken together these studies indicate that DPKQDFMRFamide-dependent enhancement of neurotransmitter release from presynaptic terminals requires activation of IP_3 receptors, presumably to release Ca^{2+} from internal stores. This is confirmed by the evidence that DPKQDFMRFamide increases peak Ca^{2+} currents in 1b boutons (Klose et al., 2010). Downstream from the Ca^{2+} increase, DPKQDFMRFamide-induced modulation of neurotransmitter release requires CaMKII. If the peptide-induced increase in neurotransmitter release was responsible for peptide's myogenic effects, one would predict that inhibition of the pathways involved in effects on the EJP amplitude and peak Ca^{2+} increase (IP_3 receptors and CaMKII) would also inhibit the ability of the peptide to increase muscle tonus. The present data indicate that this was not the case. By applying the same genetic and pharmacological approaches as those used by Dunn and Mercier (2005) and Klose et al. (2010), this study showed that inhibition of CaMKII and IP_3 receptors does not abolish the peptide's stimulatory effects on muscle tonus. In conclusion the mechanisms required for DPKQDFMRFamide's postsynaptic effects to occur are not the same as those required for presynaptic effects. This further supports the idea that peptide's stimulatory effect on muscle tonus in the absence of nerve stimulation is postsynaptic.

4.4 Conclusions and perspectives

This study provides compelling evidence that DPKQDFMRFamide acts postsynaptically via the FMRFamide G-protein coupled receptor (FR) to increase muscle tonus. However, the results also show that the response to DPKQDFMRFamide is not

completely abolished by ubiquitous silencing of the FR. This finding, together with the evidence provided from pharmacological characterization of the dFMRFamide receptor cloned and expressed in HEK and CHO cells (Johnson et al., 2003, Cazzamali and Grimmelikhuijzen, 2002), suggests that DPKQDFMRFamide might also act on receptors other than FR such as the *Drosophila* myosuppressin receptors. This seems unlikely because the physiological effects of DMS on heart and gut contractions are inhibitory, opposite to the effects of FMRFamide-containing peptides. Hence it seems unlikely that binding of DPKQDFMRFamide to DMS receptors on muscle cells would produce stimulatory effects. Further investigations are needed to reveal the relevance of the myosuppressin receptor and possible involvement of other receptors in mediating the physiological actions of DPKQDFMRFamide and other FMRFamide-containing peptides.

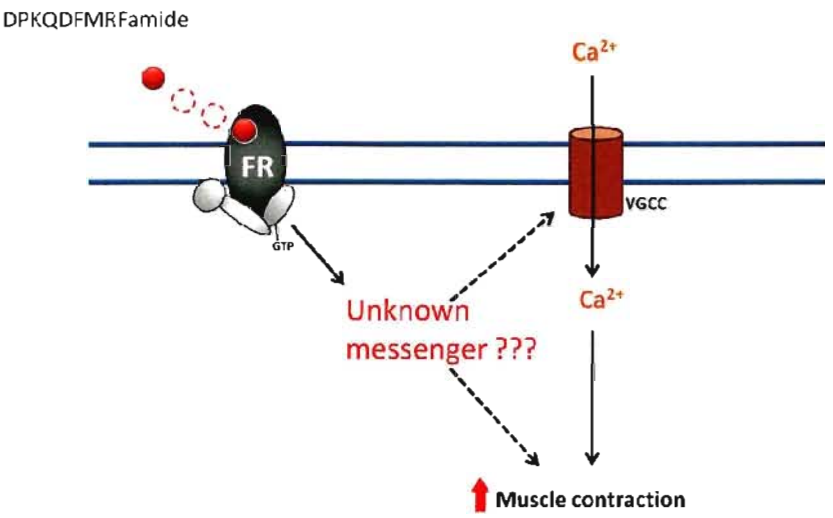
The present data indicate that cAMP, cGMP, IP_3 and CaMKII are not likely to be involved in mediating the peptide's stimulatory effect on muscle tonus. Previously it was shown that FMRFamide can reduce neurotransmitter release from *Aplysia* neurons via arachidonic acid (AA) (Piomelli et al., 1987, Taussig et al., 1989), a product of membrane phosholipid hydrolysis initiated by phospholipase A2 (PLA2). The possible involvement of AA signalling pathway in mediating effects of *Drosophila* FMRFamides remains to be examined. This opens the possibility that either AA or some unknown messenger is activated by the DPKQDFMRFRamide (Fig 15A).

The myotropic effect of DPKQDFMRFamide on larval body wall muscles does not appear to require IP_3 –dependent release of calcium from the internal stores, since larvae with disrupted PLC and IP_3 receptor function are able to respond to the peptide.

Wegener & Nässel (2000) showed that proctolin-induced contractions in the hyperneural muscle of the cockroach *Periplaneta Americana* depend greatly on the release of calcium from the sarcoplasmic reticulum via ryanodine receptors, but not on the IP_3-induced Ca^{2+} release. Hence, the possibility that the peptide increases intracellular calcium at least in part via calcium-induced calcium release through ryanodine channels has not been excluded.

The myotropic effect of DPKQDFMRFamide requires influx of calcium via voltage gated calcium channels (VGCC) (Clark et al., 2008), but the mechanisms that lead to the activation of these VGCC remain unknown. In *Drosophila* muscle tissue, both cAMP and PLC-DAG signalling molecules have been shown to modulate L-type calcium channel currents (Bhattacharya et al. 1999, Gu an Singh, 1997). Our results suggest that neither of these pathways is involved in the peptide-induced modulation of muscle contractions. The other possibility is that the peptide-activated G-protein coupled receptor is directly modulating the activity of calcium channels (Fig 15B). Studies have shown that G protein α and $\beta\gamma$ subunits can bind directly to ion channels, modulate their activity (Dascal et al., 2001, Jiang and Bajpayee, 2009) and, hence, modulate cell excitability. Further investigation is necessary to elucidate the molecular mechanisms linked to the dFMRFamide receptor.

A)



B)

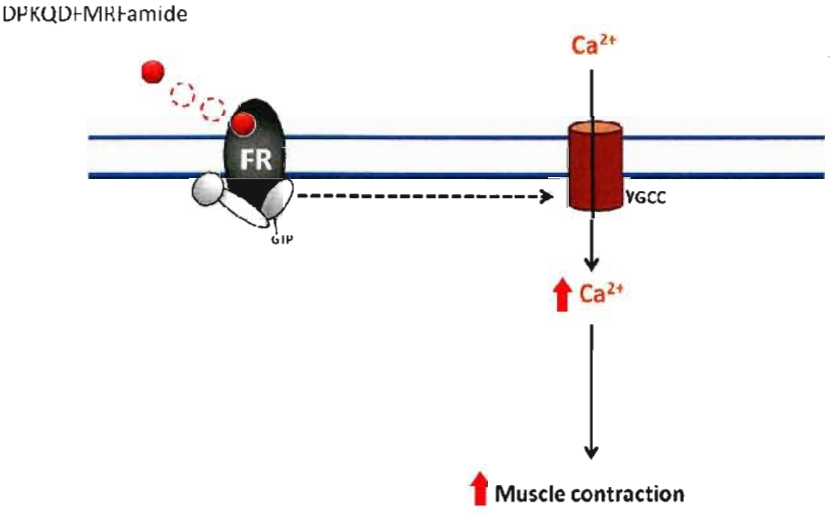


Fig. 15. Hypothetical mechanisms mediating neuropeptide modulation of *Drosophila* larval body wall muscles.

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